Tetrahydrobiopterin and Monoamine Neurotransmitter Deficiency in Mouse Models and Human Disease

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Abbreviations

AADC, Aromatic amino acid decarboxylase

AAAOH, Aromatic amino acid hydroxylase

AGMO, Alkylglycerol monooxygenase

BH_4, (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin

CNS, Central nervous system

CSF, Cerebrospinal fluid

DCoH, Dimerization cofactor of hepatocyte nuclear factor-1α

DHPR, Dihydropteridine reductase

ES cells, Embryonic stem cells

GFRP, GTP cyclohydrolase feedback regulatory protein

GMC, German Mouse Clinic

GTPCH, Guanosine triphosphate cyclohydrolase

5-HIAA, 5-hydroxyindoleacetic acid

HVA, Homovanillic acid

HPA, Hyperphenylalaninemia

5-HT, Serotonin, 5-hydroxytryptamin

5-OH-Trp, 5-Hydroxytryptophan

5-HTTLPR, 5-HTT gene-linked polymorphic promoter region

Het, Heterozygous

Homo, Homozygous

Ki, Knock-in

Ko, Knock-out

L-DOPA, L-3,4-dihydroxyphenylalanine

MAO-A, Monoamine oxidase A

NO, Nitric oxide

NOS, Nitric oxide synthase

PAH, Phenylalanine hydroxylase

PCD/DCoH, Pterin-4α-carbinolamine dehydratase

PMAT, Plasma membrane monoamine transporter

Pts-ki/ki, Homozygous *Pts*-R15C-knock-in

Pts-ki/wt, Heterozygous *Pts* -R15C-knock-in

Pts-ko, Homozygous *Pts*-knock-out

Pts-ko/wt, Heterozygous *Pts* -knock-out

PTPS, 6-Pyruvoyl-tetrahydropterin synthase

qBH_2, Quinonoid dihydrobiopterin

SERT, Serotonin re-uptake transporter

SR, Sepiapterin reductase

STin2VNTR, Serotonin transporter intron 2 VNTR

TH, Tyrosine hydroxylase

TPH, Tryptophan hydroxylase

VNTR, Variable number of tandem repeat

Wt, wt/wt, wild-type

Summary

The present dissertation is based on different aspects within tetrahydrobiopterin (BH_4) and monoamine neurotransmitter deficiency in mouse models and human disease. Thus, it is subdivided into two parts covering (A) new animal models to study the pathophysiology and potential treatment options in mice deficient for BH_4 (chapters 2 and 3), as well as (B) human genetic studies in subjects with low brain serotonin, autism spectrum disorders (ASDs) and serotonin re-uptake transporter mutations (chapters 4 and 5).

(A) New mouse model to study BH_4 deficiency by targeting a biosynthetic (Pts) and cofactor regulatory (Gchfr) gene

Previous studies have shown that homozygous *Pts*-knock-out (*Pts*-ko/ko) mice exhibit newborn lethality (whereas *Pts*-ko/wt mice had no phenotype). Thus, the generation of a new and suitable mouse model to study human BH_4 deficiency was essential. One goal of this study was to generate two new *Pts* mouse models to study PTPS deficiency: a homozygous *Pts*-R15C-knock-in (*Pts*-ki/ki) and a compound heterozygous *Pts*-R15C-ki/*Pts*-ko (*Pts*-ki/ko). After successful generation, the biochemical characterization of the *Pts*-ki/ki mice revealed no hyperphenylalaninemia (HPA) or brain monoamine neurotransmitter deficiency, but reduced PTPS enzyme activity and elevated neopterin in liver and brain. Despite that *Pts*-ki/ki mice were found not to be a suitable mouse model to study BH_4 deficiency, they displayed compared to wild type mice an abnormal fat distribution, and unexpected and progressive weight gain when exposed for several weeks to either standard chow or high fat diet.

Breeding of *Pts*-ki/ko mice led to two different phenotypes depending on the maternal genotype: one lethal 3-4 days after birth (“affected”) and one which survived (“unaffected”) until sacrificed at 3 weeks of age. The biochemical characterization of the “affected” *Pts*-ki/ko mice, which were born from *Pts*-ki/ki mothers crossed with *Pts*-ko/wt fathers, demonstrated reduced PTPS enzyme activity and biopterin in liver and brain, elevated neopterin in liver and brain, mild HPA as well as brain monoamine neurotransmitter deficiency. In contrast, “unaffected” *Pts*-ki/ko mice, which were born from *Pts*-ko/wt mothers crossed with *Pts*-ki/ki fathers, revealed reduced PTPS enzyme activity in liver and brain but normal levels of blood phenylalanine, biopterin (liver and brain), neopterin (liver and brain) and brain monoamine neurotransmitters. Therefore, “affected” *Pts*-ki/ko mice had all biochemical hallmarks of BH_4 deficiency, and were thus found to be a suitable mouse model to study BH_4 deficiency.

Furthermore, it is our hypothesis that the severe BH_4 deficiency observed in the "affected" *Pts*-ki/ko F1-offspring mice is due to limited BH_4 supply during early life phase by the *Pts*-ki/ki mothers.

To study the *in vivo* role of the BH_4-regulatory protein GFRP in liver and brain, as well as its potential contribution to human diseases, we aimed at generating the first animal model for GFRP, a conditional knock-out by targeted mutagenesis of the murine *Gchfr* gene. Successful targeting event in ES cells followed by morula aggregation led to the generation of four chimeras with up to 80 % chimerism. Unfortunately, *Gchfr*-floxed heterozygous mice could not be generated due to unsuccessful germline transmission. In the following, we decided to test a new and improved targeting vector. This alternative targeting vector was recently linearized and electroporated into ES cells derived from the C57BL/6 strain.

(B) Low brain serotonin, ASDs and serotonin re-uptake transporter mutations

In a previous study from our laboratory, five boys were described with motor delay, hypotonic-ataxic, learning deficits and short attention span. The catecholamine and serotonin neurotransmitter were investigated in the cerebrospinal fluid (CSF) due to the neuro-developmental abnormalities. The metabolic analysis of the CSF revealed isolated low serotonin end-metabolite 5-hydroxyindoleacetic acid (5-HIAA), which suggested a down-regulation of the serotonin turnover. For that reason, the five patients received daily treatment with serotonin precursor 5-hydroxytryptophan and AADC inhibitor carbidopa, which led to clinical improvements and normalization of the 5-HIAA levels in CSF and urine.

To investigate the underlying genetic aspects responsible for the low brain serotonin and the neuro-developmental abnormalities, I performed a genetic candidate gene approach within all five affected boys. Since it was hypothesized that dysfunction of the brain serotonin development system contributes to the observed phenotype, the coding sequences of the serotonin re-uptake transporter gene *SLC6A4*, encoding SERT, and of the BH_4 regulatory gene *GCHFR*, encoding GFRP, were investigated first. GFRP is the only gene in the BH_4 metabolism for which no human deficiency has been descried. No mutations were found within the *GCHFR* gene, but in one patient I found the heterozygous p.G56A mutation in the *SLC6A4* gene, which previously has been shown to be associated with autism and rigid-compulsive behaviour. In retrospect, this patient was diagnosed with atypical autism and speech delay when subjected to the diagnostic criteria for autistic disorder according to DSM-IV. Altogether, this study showed for the first time an association between low CNS serotonin, atypical autism and heterozygous SERT-G56A allele, suggesting that patients with

atypical autism, carrying the heterozygous SERT-G56A mutation, may respond to treatment with 5-hydroxytryptophan and carbidopa by clinical improvement and normalization of serotonin turnover in the brain. Moreover, these findings suggested for the first time the metabolic and underlying genetic abnormalities for atypical autism.

The novel findings encouraged me to investigate a cohort of 125 ASDs patients with either unknown, normal or isolated low 5-HIAA in the CSF using a genetic candidate gene approach involving besides *SLC6A4* and *GCHFR*, the serotonin re-uptake transporter gene *SLC29A4* encoding PMAT. In this cohort, I found five patients carrying the known heterozygous p.G56A mutation in the *SLC6A4* gene. Again, no patients were found with mutations in the *GCHFR* gene. Moreover, I identified for the first time patients with mutations in the *SLC29A4* gene: in 9 ASD patients I found one of the following heterozygous mutations: p.M24L, p.D29G, p.A138T and p.D326E. Moreover, they were found to be negative in an independent group of at least 300 control subjects. One patient was found to carry heterozygous mutations in both genes, *SLC6A4* (p.G56A) and the *SLC29A4* (p.D326E).

By investigating the function and location of the two PMAT-mutant alleles p.A138T and p.D326E, which were found in patients diagnosed with isolated low 5-HIAA and ASDs, we found that both PAMT mutant transporters, 138T and 326E, exhibited reduced transport activities for serotonin and dopamine, and that they were located in the plasma membrane of the transiently transfected (MDCK) cells, similar to wild-type PMAT.

In a next step, whole exome sequencing was performed on the two patients carrying the heterozygous PMAT alteration p.A138T or p.D326E (and SERT-Gly56Ala) plus their healthy parents and sister to potentially understand the genetic link between isolated low 5-HIAA in CSF, ASDs and serotonin re-uptake transporter mutations.

Based on a hypothetic “genetic accumulation-model” for ASD, we specifically selected for non-synonymous amino acid substitutions within all reported serotonin-related and/or autism-associated candidate genes, and with a mutant-allele frequency of <1. The cumulative genetic burden using such a filtering strategy, revealed a tendency towards more “serotonin gene hits” in the two ASD affected patients, as compared to the other family members, indicating that alterations in mainly serotonin homeostasis genes might be responsible for the autistic phenotype. Furthermore, our exome sequencing data support the hypothesis that ASDs may be caused by a certain (threshold) number of individual heterozygously mutated genes associated with serotonin homeostasis.

Zusammenfassung

Die vorliegende Dissertation befasst sich mit verschiedenen Aspekten des Tetrahydrobiopterin (BH_4)- und Monoamin-Neurotransmitter-Mangels in Maus-Modellen und beim Menschen. Die Resultate sind in zwei Teile gegliedert, (A) neue Tiermodelle zur Untersuchung der Pathophysiologie und potentiellen Behandlungsmöglichkeiten des BH_4-Mangels in Mäusen (Kapitel 2 und 3) und (B) genetische Untersuchungen von Patienten mit deutlich erniedrigtem Serotonin im ZNS, Autismusspektrum-Störung (ASD) und Mutationen in Serotonin-Reuptake Transporter-Genen.

(A) Neue Mausmodelle zur Untersuchung des BH_4-Mangels durch gezielte Mutagenese eines biosynthetischen (Pts) und eines Kofaktor-regulatorischen (Gchfr) Gens

Vorangegangene Experimente mit transgenen Mäusen haben gezeigt, dass sich ein homozygoter *Pts*-knock-out (*Pts*-ko/ko) postnatal letal auswirkt (hingegen sind Pts-ko/wt Mäuse phänotypisch unauffällig). Somit bestand die Notwendigkeit für die Generierung neuer Tiermodelle zur Untersuchung des BH_4-Mangels beim Menschen. Ein Ziel dieser Arbeit bestand darin, zwei *Pts*-Mausmodelle zur Untersuchungen des BH_4-Mangels herzustellen, eine homozygote *Pts*-R15C-knock-in (*Pts*-ki/ki) und eine „compound“ heterozygote *Pts*-R15C-ki/*Pts*-ko (*Pts*-ki/ko) Mausmutante. Nach erfolgreicher Generierung dieser Mausmutanten ergaben biochemische Untersuchungen bei der homozygoten *Pts*-ki/ki Maus keine Anzeichen von Hyperphenylalaninämie (HPA) oder Monoamin-Neurotransmittermangel, hingegen zeigten die Tiere reduzierte Enzymaktivitäten für die 6-Pyruvolytetrahdropterin Synthase (PTPS) und erhöhte Neopterin-Werte in Leber und Gehirn. Obwohl die *Pts*-ki/ki Mutante kein geeignetes Modell für den BH_4-Mangel darstellte, hatten diese Mäuse im Vergleich zur Wildtypkontrolle eine abnorme Fettverteilung sowie eine ungewöhnliche Gewichtszunahme nach Fütterung über mehrere Wochen mit normalem oder fettangereichertem Futter.

Die Generierung von heterozygoten *Pts*-ki/ko Mäusen führte zu zwei unterschiedlichen Phänotypen, abhängig vom Genotyp der Mutter: während Mäuse in einer Gruppe 3-4 Tage nach der Geburt verstarben (so genannte „betroffene“), überlebten die Tiere einer zweiten Gruppe problemlos (so genannte „nicht betroffene“) bis sie im Alter von 3 Wochen für Untersuchungszwecke euthanasiert wurden. Die biochemische Charakterisierung der „betroffenen“ *Pts*-ki/ko Mäuse, Nachkommen von *Pts*-ki/ki Müttern gepaart mit *Pts*-ko/wt Vätern, zeigte verminderte PTPS-Enzymaktivitäten und Biopterinwerte in Leber und Gehirn,

erhöhte Neopterinwerte in Leber und Gehirn, eine milde HPA sowie einen Monoamin-Neurotransmittermangel im Gehirn. Im Gegensatz dazu zeigten „nicht betroffene“ *Pts*-ki/ko Mäuse, Nachkommen von *Pts*-ko/wt Müttern gepaart mit *Pts*-ki/ki Vätern, eine reduzierte PTPS-Aktivität in Leber und Gehirn, jedoch normale Phenylalaninwerte im Blut sowie normale Werte für Biopterin (Leber und Hirn), Neopterin (Leber und Hirn) und Monoamin-Neurotransmitterwerte im Gehirn. Demzufolge sind die „betroffenen“ *Pts*-ki/ko Mäuse ein geeignetes biochemisches Modell zur Untersuchung des BH_4-Mangels. Weiter spekulieren wir, dass der schwere BH_4-Mangel bei den „betroffenen“ *Pts*-ki/ko Mäusen durch eine begrenzte Verfügbarkeit von BH_4 in der Muttermilch der *Pts*-ki/ko Mütter während der frühen Lebensphase der Nachkommen verursacht wird.

Um die *in vivo* Rolle des regulatorischen „feed-back regulatory protein“ GFRP in Leber und Gehirn sowie die potentielle Rolle des GFRP-Mangels beim Menschen zu untersuchen, sollte eine konditionelle *knock-out* Maus durch gezielte Mutagenese des murinen *Gchfr* Gens erzeugt werden. Nach erfolgreichem Gen-Targeting in ES-Zellen und Morula-Aggregation wurden vier Mäuse mit bis zu 80%-igem Chimärismus geboren. Es wurde darum beschlossen, einen verbesserten Targeting-Vektor zu konstruieren und in ES-Zellen zu testen. Mit Hilfe dieses alternativen Targeting-Vektors wurde kürzlich eine erfolgreiche Elektroporation in C57BL/6-ES-Zellen durchgeführt.

(B) Abnormtiefes Serotonin im ZNS, Autismusspektrum-Störung (ASD) und Mutationen in Serotonin-Reuptake Transporter-Genen

In einer vorangegangenen Studie wurden fünf Knaben mit motorischer Retardierung, hypotoner Ataxie, Lerndefizit und kurzer Aufmerksamkeitsspanne beschrieben. Die Metabolite der Katecholamin- und Serotonin-Neurotransmitterbiosynthese wurden im Liquor untersucht (auch Zerebrospinal-Flüssigkeit). Diese Analyse zeigte deutlich erniedrige Werte für den Serotonin-Metaboliten 5-Hydroxyindolessigsäure (5-HIAA), was auf eine erniedrigte Regulation des Serotoninumsatzes schliessen liess. Aus diesem Grund erhielten die fünf Patienten eine tägliche Behandlung mit der Serotoninvorstufe 5-Hydroxytryptophan (plus dem Decarboxylase-Hemmer „Carbidopa“), was zu einer klinischen Verbesserungen und Normalisierung der 5-HIAA im Liquor und im Urin führte.

Zur weiteren ursächlichen Abklärung der abnormtiefen Serotoninwerte im Gehirn und der neuronalen Entwicklungsstörungen dieser Knaben wurde eine Kandidaten-Gen-Analyse durchgeführt, basierend auf der Hypothese, dass Funktionsstörungen des Serotonin-

Gleichgewichtszustandes („Serotonin-Homöostase“) vorliegen. Im Folgenden wurden die kodierenden Sequenzen des Serotonin-Reuptake-Gens *SLC6A4*, das für das SERT Protein kodiert, und für das *GCHFR* Gen, welches für das BH_4-regulatorische Protein GFRP kodiert, untersucht. Das *GCHFR* Gen ist das einzige Gen im BH_4-Metabolismus, für das bislang kein Defekt beim Menschen beschrieben wurde.

Es wurden keine Mutationen im *GCHFR* gefunden, hingegen wurde in einem der untersuchten Patienten die heterozygote p.G56A Mutation im Gen *SLC6A4* nachgewiesen. Dieses gut untersuchte Mutanten-Allel wurde in verschiedenen Studien mit Autismus und zwanghaftem Verhalten assoziiert. Weiter wurde retrospektiv bei dem von der Mutation betroffenen Patienten ein atypischer Autismus und eine Sprachverzögerung diagnostiziert (gemäss den diagnostischen Kriterien zur Autismus-Diagnose nach DSM-IV). Die hier vorgelegte Studie zeigt somit zum ersten Mal einen Zusammenhang zwischen abnormtiefem Serotonin im ZNS, Autismusspektrum-Störung (ASD) und der heterozygoten p.G56A Mutation in einem Serotonin-Reuptake Transporter-Gen. Weiter reagiert dieser Patient mit atypischem Autismus auf die Behandlung mit 5-Hydroxytryptophan (und Carbidopa) mit einer klinischen Besserung und Normalisierung der Serotoninmetabolite im ZNS. Zusammenfassend weisen diese Ergebnisse zum ersten Mal auf einen möglichen (Serotonin-) Stoffwechseldefekt als genetische Ursache für einen atypischen Autismus hin.

Die neuen Erkenntnisse ermutigten mich, eine Kohorte von 125 Patienten mit ASD und normalem oder abnormtiefen 5-HIAA in der Zerebrospinalflüssigkeit auf Mutationen im *SLC6A4* und *GCHFR* Gen, sowie dem *SLC29A4* Gen, das für den Serotonin-Reuptake Transporter PMAT kodiert, zu untersuchen. In dieser Gruppe fand ich fünf Patienten mit der bekannten heterozygoten p.G56A Mutation im *SLC6A4* Gen. Wie zuvor hatte keiner dieser Patienten eine Mutation im *GCHFR* Gen. Hingegen fand ich zum ersten Mal Patienten mit Mutationen im *SLC29A4* Gen: in 9 ASD Patienten wurden folgende heterozygote Varianten nachgewiesen: p.M24L, p.D29G, p.A138T und p.D326E. Gleichzeitig kommen diese Mutationen in einer unabhängigen Kontrollgruppe von mindestens 300 Individuen nicht vor. Einer der Patienten wies gleichzeitig eine Mutation im *SLC6A4* Gen (SERT-G56A) und im Gen *SLC29A4* auf (PMAT-D326E).

In der Folge wurde die Funktion der zwei PMAT-Mutanten-Allele A138T und D326E, die beide bei je einem Patienten mit tiefem 5-HIAA im Liquor und ASD diagnostiziert wurden, charakterisiert. Beide Transportermutationen, 138T und 326E, hatten reduzierte Transportaktivitäten für Serotonin und Dopamin. Weiter wurde nachgewiesen, dass die

beiden PMAT-Mutationen, ähnlich wie das Wildtyp PMAT Protein, in der Plasmamembran von transient transfektierten (MDCK) Zellen lokalisiert sind.

In einem nächsten Schritt wurde eine komplette Exom-Analyse bei den beiden Patienten, welche die heterozygoten PMAT Mutationen p.A138T und p.D326E (sowie die SERT-G56A Mutation) trugen, durchgeführt. Als Kontrollen wurden je deren gesunde Eltern und je ein Geschwister analysiert. Das Ziel war, eine mögliche genetische Verbindung zwischen Serotonin-Reuptake Transporter Mutationen, tiefen 5-HIAA-Werten im Liquor in der Zerebrospinalflüssigkeit und ASD zu finden.

Basierend auf einem hypothetischen "genetic accumulation-model" für ASD wurden nur „non-synonymous“ Aminosäurenaustausche von allen bekannten Genen, die ein bekannte Assoziation mit Serotonin Homöostase und/oder ASD-assoziierte Kandidatengene zeigten und eine Mutanten-Allel-Frequenz von <1 aufwiesen, selektiert. Die aus dieser Such-Strategie resultierende kumulative genetische Belastung ergab eine Tendenz zu vermehrten "Serotonin-Gen-Hits" in den beiden ASD Patienten im Vergleich zu den anderen Familienmitgliedern. Dies weist darauf hin, dass in erster Linie Veränderungen in Genen der Serotonin-Homöostase für den autistischen Phänotyp verantwortlich sein könnten. Weiter unterstützen unsere Resultate zur Exom-Sequenzierung die Hypothese, dass ASD durch eine bestimmte (Schwellen-) Zahl von einzelnen heterozygot mutierten Genen, die mit der Serotonin Homöostase assoziiert sind, verursacht werden könnte.

1

Introduction

1 Introduction

(6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) is an essential cofactor for several enzymes in the human metabolism. Among these are the aromatic amino acid hydroxylases (AAAOH): phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), all three isoforms (neuronal, inducible, and endothelial) of nitric oxide synthases (NOS) and alkylglycerol monooxygenase (AGMO) (Figure 1). Hence, BH_4 plays a central role in the metabolism involving phenylalanine degradation, monoamine neurotransmitter biosynthesis and nitric oxide production. Consequently, BH_4 deficiency leads to dopamine and serotonin monoamine neurotransmitter deficiency, reduced NO production, and often but not always to hyperphenylalaninemia (HPA). The requirement of BH_4 is reflected by the severe disturbance or even lethality in the case of cofactor limitation due to mutations in human BH_4-metabolic genes.

1.1 *De novo* biosynthesis of Tetrahydrobiopterin (BH_4)

BH_4 can be synthesized by two distinct pathways: 1) the *de novo* pathway uses guanosine triphosphate (GTP) as a precursor, and 2) the salvage pathway depends on pre-existing 7,8-dihydropterin (Blau et al., 2001, Thöny et al., 2000).

In the *de novo* biosynthetic pathway BH_4 is generated from GTP by three enzymatic reactions (Figure 1). The first and rate-limited enzyme is GTP cyclohydrolase I (GTPCH) which catalyzes the conversion of GTP to 7,8-dihydroneopterin triphosphate (NH_2TP). The GTPCH reaction requires Zn^{2+} and involves two hydrolyses and an Amadori rearrangement of the ribose moiety, yielding formic acid as a by-product (Blau et al., 2001, Thöny et al., 2000) (Figure 1). The second enzyme in the *de novo* pathway is 6-pyruvoyl-tetrahydropterin synthase (PTPS), which converts NH_2TP to 6-pyruvoyl-tetrahydropterin (PPH_4) by a two-step reaction involving an internal redox transfer and triphosphate elimination in a Mg^{2+}-and Zn^{2+}-dependent reaction (Blau et al., 2001, Thöny et al., 2000) (Figure 1). The third and last enzyme is sepiapterin reductase (SR), which requires NADPH for the convention of 6-pyruvoyltetrahydropterin to BH_4. A two-step reduction is necessary to convert both side-chain keto groups into hydroxyl functions with proper stereochemistry (Blau et al., 2001, Thöny et al., 2000) (Figure 1).

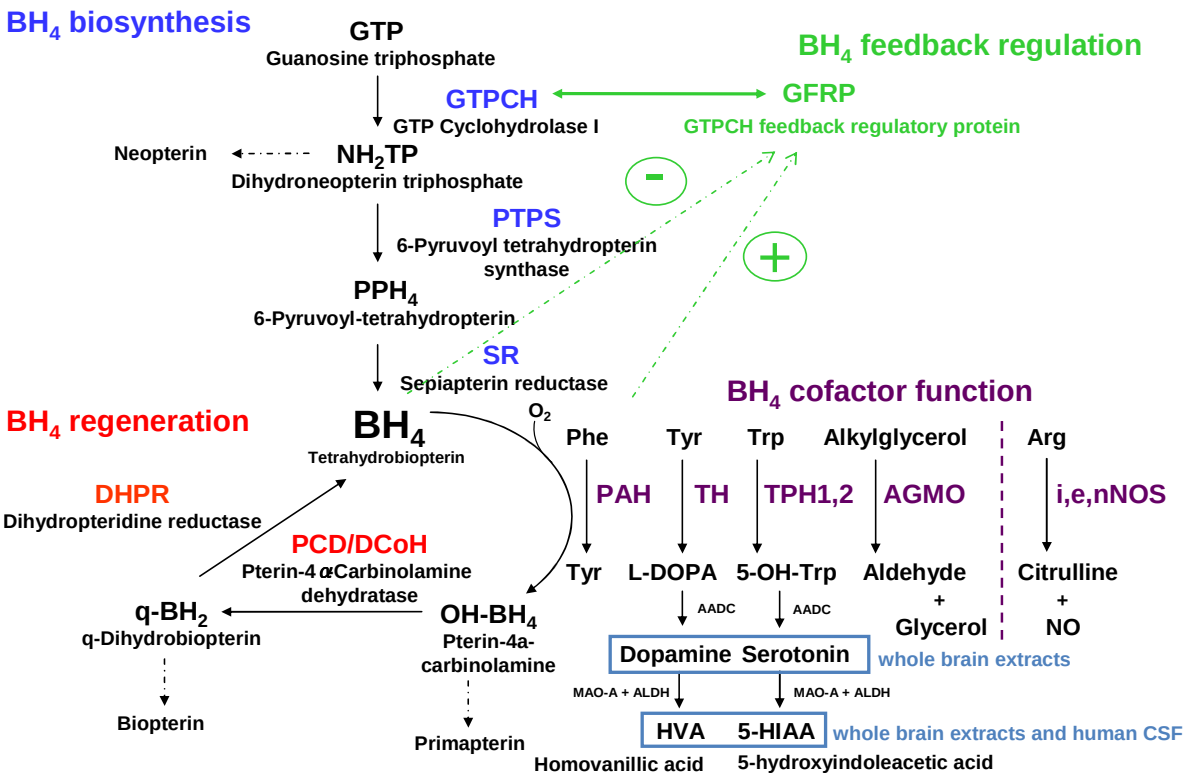

Figure 1: BH_4 *de novo* biosynthesis and BH_4-dependent metabolism

The (6R)-L-erythro-5,6,7,8-tetrahydrobiopterin (BH_4) cofactor is synthesized from guanosine triphosphate (GTP) by a set of reactions involving three enzymes: GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR). The complete hydroxylating system of aromatic amino acids consists of the two additional BH_4-regenerating enzymes namely pterin-4α-carbinolamine dehydratase (PCD/DCoH) and dihydropteridine reductase (DHPR). The presence of the amino acid phenylalanine (Phe) or cofactor BH_4 itself is thought to regulate cofactor biosynthesis in liver via the GTP cyclohydrolase I feedback regulatory protein (GFRP) mainly through its protein-super complex with GTPCH. One of the best investigated function of BH_4 is its action as a natural cofactor for the aromatic amino acids hydroxylase (AAAOH), i.e. phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase type 1 and 2 (TPH1, peripheral and TPH2, neuronal). Moreover, BH_4 is an essential cofactor for all three isoforms of nitric oxide synthase (inducible, endothelial, neuronal NOS) as well as alkylglycerol monooxygenase (AGMO). The various colours represent enzymes for BH_4 biosynthesis (blue), BH_4 regeneration (red), BH_4 cofactor function (purple) and BH_4 feedback regulation (green). The dash line indicates that BH_4 as a cofactor for all three isoforms of NOS is not oxidized to pterin-4a-carbinolamine (OH-BH_4) but instead is converted into a pterin cation radical (not shown). Note the differences for detecting monoamine neurotransmitter metabolites in the CNS between mouse (whole brain extracts) and humans (CSF) are indicated by the light-blue boxes. Phe: Phenylalanine, Tyr: Tyrosine, L-DOPA: L-3,4-dehydroxyphenylalanine, Trp: Tryptophan, 5-OH-Trp: 5-hydroxytryptophan, AADC: aromatic amino acid decarboxylase, MAO-A: monoamine oxidase A, Arg: Arginine, NO: nitric oxide.

1.2 Regeneration of BH_4

The three AAAOHs require two electrons, molecular oxygen and iron for the enzymatic hydroxylate of their amino acid substrates Phe, Tyr and Trp. One electron is transferred to Fe^{3+} in the active site of the AAAOH causing a reduction to Fe^{2+} which has been linked to the formation of an active AAAOH and the other electron is transferred to molecular oxygen, which during the catalytic event of the AAAOH, is transferred to the corresponding amino acid. Both electrons are provided by the cofactor BH_4 and as a consequence, BH_4 is oxidized to its hydroxyl compound pterin-4α-carbinolamine (also known as 4α-Hydroxy-BH_4, BH_4-4a-carbinolamine and BH_4-OH) (Figure 1) (Thöny et al., 2000). Two enzymes are responsible for the dehydration and reduction of pterin-4α-carbinolamine back to BH_4 namely pterin 4α-carbinolamine dehydratase (PCD) as well as dihydropteridine reductase (DHPR), respectively (Thöny et al., 2000).

In the BH_4 regeneration, PCD catalyzes the dehydration of pterin-4α-carbinolamine to quinonoid dihydrobiopterin (q-BH_2) by elimination of one molecule of water from the oxidized pterin. Besides the role of PCD in the BH_4 regeneration, PCD is also a cofactor that regulates dimerization of the mammalian homeodomain protein HNF-1α thus PCD is also known as dimerization cofactor of hepatocyte nuclear factor-1α (DCoH) (Mendel et al., 1991). The final conversion of q-BH_2 into BH_4 is carried out by NADH and the second enzyme dihydropteridine reductase (DHPR) (Thöny et al., 2000) (Figure 1).

In the absence of PCD/DCoH and DHPR (e.g. in patients with PCD/DCoH deficiency or DFPR deficiency), pterin-4α-carbinolamine and q-BH_2 are non-enzymically converted to 7-dihydrobiopterin (primapterin) and 7,8-dihydrobiopterin (biopterin), respectively which are both excreted in the urine (Thöny et al., 2000).

The enzymatic recycling or regeneration of BH_4 is necessary for two reasons: first to ensure a continuous supply of reduced cofactor and second to prevent accumulation of harmful metabolites produced by rearrangement of pterin-4α-carbinolamine (Thöny et al., 2000).

As a cofactor for NOS, BH_4 is not oxidized to pterin-4α-carbinolamine but instead, during the transfer of electrons to the ferrous-dioxygen complex in the oxygenase domain, converted into a pterin cation radical ($BH_3^{\bullet}H^+$ or $BH_4^{+\bullet}$) (Werner et al., 2003, Wei et al., 2003, Schmidt and Alp, 2007). Subsequently, the pterin cation radical returns to the BH_4 state after each electron transfer step thus BH_4 regeneration by PCD/DCoH and DHPR is not a requirement for NOS activity (Schmidt and Alp, 2007, Thöny et al., 2000).

1.3 Regulation of BH_4 biosynthesis

1.3.1 BH_4 regulates its biosynthesis primarily via GTPCH

GTPCH, the first and rate-limiting enzyme of the biosynthesis of BH_4 which catalyses the conversion of GTP to H_2NTP, is the major controlling point of the BH_4 production (Thöny et al., 2000, Werner-Felmayer et al., 2002). Changes in GTPCH activity are directly correlated with changes in the BH_4 levels and its activity is predominantly regulated at the transcriptional level by a variety different stimuli such as proinflammatory cytokines like interferon-γ (Gesierich et al., 2003), interleukin-1β, TNF-α, INF-α and β (Milstien et al., 1996). Moreover, administration of bacterial lipopolysaccharide (LPS) to rats have shown to increase GTPCH activity and subsequently the BH_4 levels within the cerebellum, liver, spleen and adrenal gland (Milstien et al., 1996, Werner-Felmayer et al., 2002). Additionally, certain hormones (e.g. follicle stimulating hormone), growth factor (e.g. epithelial growth factor (EGF)) or neuronal growth factors (NGF)), cAMP, the vasodilatory peptide adrenomedullin and insulin have been found to induce GTPCH expression (Werner-Felmayer et al., 2002, Ishii et al., 2001). Likewise, several stimuli have been shown to down-regulate GTPCH expression among these are the anti-flammatory cytokines IL-4, IL-10, TGF-β and the neurohormone melatonin (Werner-Felmayer et al., 2002). GTPCH activity has also been reported to be modulated at the post-transcriptional level by phosphorylation (Imazumi et al., 1994, Lapize et al., 1998, Ishii et al., 2001).

1.3.2 GTP cyclohydrolase feedback regulatory protein (GFRP)

In vitro studies on rat liver extracts have shown that BH_4 regulates its own biosynthesis through feedback inhibition of GTPCH and that this end-product feedback inhibition is mediated through protein complex formation with GTP cyclohydrolase feedback regulatory protein (GFRP) (Harada et al., 1993) (Figure 1).

Furthermore, structural based studies have demonstrated that feedback inhibition of the GTPCH-GFRP complex also can be induced by dihydrobiopterin (BH_2) (Maita et al., 2004).

$_L$-phenylalanine has been shown to reverse the BH_4-mediated feedback inhibition as it changes the inactive GTPCH-GFRP complex to its active form (Yoneyama et al., 1997) (Figure 1). Hence, the activity of GTPCH is positively/stimulatory regulated in presence of the amino acid phenylalanine and negatively (inhibitory) by the cofactor BH_4 itself. Both the positive and negative feedback regulation takes place GFRP through a reversible protein-protein interaction with GTPCH (Maita et al., 2002).

Cross-linking experiments have shown that both the stimulatory and inhibitory GTPCH-GFRP complexes consist of two pentamers of GFRP interacting with one decamer of GTPCH in a face-to-face manner (Yoneyama and Hatakeyama, 1998, Maita et al., 2002). The phenylalanine-mediated feed-forward stimulation via GFRP not only regulates the BH_4 biosynthesis but also provides a mechanism to regulate phenylalanine catabolism and to avoid toxic accumulation of phenylalanine (Bader et al., 2001). This may explain the high plasma BH_4 concentration observed in patients with hyperphenylalaninemia (HPA) caused by PAH deficiency (Ponzone et al., 1993).

GFRP has been purified, sequenced and cloned from rat liver (Milstien et al., 1996, Bader et al., 2001, Yoneyama et al., 1997). The known GFRP sequences are highly homologous, suggesting that the interaction between GTPCH and its regulatory protein are similar in many species (Bader et al., 2001). The rat GFRP protein consists of 83 amino acid residues and is a pentamer of 50 kDa with identical subunits (Yoneyama and Hatakeyama, 1998, Yoneyama et al., 1997, Bader et al., 2001). Crystral structure of rat GFRP has revealed that each of the five subunits in GFRP is folded into a α/β-structure with a dominant six-stranded anti-parallel β-sheet and that the five subunits form a propeller-like pentamer with helix H2 located in the interface between the subunits (Bader et al., 2001).

Like GTPCH, GFRP is subjected to transcriptional regulation which contributes to changes in both the GTPCH activity and subsequently the BH_4 levels. Several studies have demonstrated that GFRP mRNA expression is altered in response to different stimuli like bacterial LPS and hydrogen peroxide (Gesierich et al., 2003, Kalivendi et al., 2005, Werner et al., 2002) thus suggesting that BH_4 biosynthesis can be independent of metabolic control by phenylalanine (Werner et al., 2002).
GFRP mRNA studies by Northern blot analysis and *in situ* hybridization have revealed that the expression pattern in rat tissues correlates with that of GTPCH, i.e. GFRP has been found to be expressed in the brain but also in peripheral organs such as liver, kidney, heart, lung, skeletal muscle, spleen, testis (Milstien et al., 1996, Kapatos et al., 1999).

In contrast to the liver, the regulation of BH_4 biosynthesis in brain is not yet known, but GFRP appears to be specifically expressed in some brain areas or cell types as indirect evidence from studies with primary neuronal cultures which have indicated that GFRP regulates BH_4 biosynthesis within serotonergic neurons but not within dopaminergic neurons (Kapatos et al., 1999).

Beside the regulation of the BH_4 biosynthesis via GTPCH either at the transcriptional level, the posttranscriptional level or by the action of BH_4 (feedback inhibition) and phenylalanine (feedforward stimulation), the BH_4 biosynthesis has also been shown to be regulated via PTPS as cell-type-specific exon 3 skipping in the PTPS pre-mRNA have been shown to cause a premature stop codon and thereby influencing the *PTS* gene expression (Leitner et al., 2003, Werner et al., 2011). Moreover, phosphorylation of PTPS has been shown to be essential for normal protein activity (Scherer-Oppliger et al., 1999).

1.4 BH_4 cofactor function

BH_4 is an essential cofactor for several enzymes, among them the three AAAOHs: 1) PAH, which is responsible for the degradation of the tyrosine to phenylalanine in the liver, 2) TH, which is the rate-limiting enzyme in the biosynthesis of catecholamines (e.g. dopamine) and 3) TPH1, peripheral and TPH2, neuronal (Walther and Bader, 2003), which are the rate-limiting enzyme in the 5-hydroxytryptamine (serotonin) biosynthesis (Thöny et al., 2000). Moreover, BH_4 is cofactor for all three isoforms (neuronal, inducible, and endothelial) of NOS as well as AGMO (previous identified as GEMO or ether lipid oxidase) (Wei et al., 2003, Watschinger et al., 2010, Werner et al., 2003) (the function of NOS and AGMO are breifly described below).

For that reason, BH_4 plays an important role in the human metabolism involving phenylalanine degradation, monoamine neurotransmitter biosynthesis and nitric oxide (NO) production.

The BH_4-dependent NOSs catalyze two sequential monooxygenase reactions: $_L$-arginine (Arg) is first hydroxylated to generate N-hydroxy-$_L$-arginine as an enzyme-bound intermediate, which is further oxidized in a second reaction to generate nitric oxide (NO) and citrulline (Wei et al., 2003). NO plays an important role in the regulation of vascular tone by mediating endothelium-dependent vasodilatation.

Furthermore, NO is a potent inhibitor of platelet and leukocyte adhesion to the vascular wall and inhibits smooth muscle cell proliferation (Gesierich et al., 2003). There is a positive correlation between the availability of BH_4 and the biosynthesis of NO in endothelial cells and smooth muscle cells (Gesierich et al., 2003). When BH_4 is limited in the endothelial cells, the electron transferred from eNOS flavins (flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN)) becomes uncoupled to Arg oxidation, the ferrous-dioxygen dissociates and superoxide (O_2^-) is produced from the oxygenase domain.

Superoxide generation by eNOS due to limited BH_4 has been observed in several vascular diseases including diabetes, cigarette smoking, hypertension, chronic nitrate tolerance and overt atherosclerosis (Schmidt and Alp, 2007).

AGMO is the only enzyme known to cleave the O-alkyl ether bond in alkylglycerols, yielding an aldehyde and a glycerol derivate (Watschinger et al., 2010). The aldehyde is detoxified by conversion to the corresponding acid by long-chain fatty aldehyde dehydrogenase. BH_4 leaves the reaction as q-BH_2 which is subsequently recycled back to BH_4 by DHPR (Watschinger et al., 2010).

Beside, the essential cofactor function for the three AAAOHs, the three NOSs and AGMO, BH_4 has been found to be involved in other less defined cellular functions such as cell proliferation, differentiation, neuroprotection, neurotransmitter release, neuropathic and inflammatory pain, dopaminergic neurodegeneration in Parkinson's disease (Choi et al., 2006, Koshimura et al., 2000, Tegeder et al., 2006). Several experiments with PC12 cells have demonstrated how the decision whether a cell proliferates or dies, either due to apoptosis or necrosis, is influenced by BH_4 (Werner-Felmayer et al., 2002). Moreover, it has been reported that 6R-BH_4 content in CSF decreases in aged people suggesting a role of 6R-BH_4 for aging (Koshimura et al., 2000).

Additionally, BH_4 biosynthesis has also been found to be important in cells that do not contain the AAAOHs for instance lymphocytes where the production of biopterins are significantly increased during immune activation (1988).

1.5 Human BH_4 deficiencies

Human BH_4 deficiencies, previously termed "atypical PKU", are a highly heterogeneous group of rare progressive neurological disorders ranking from mild forms without any treatment to more severe forms which require life-long treatment (Shintaku et al., 1988). BH_4 deficiencies are caused by autosomal recessively inherited (expect for the autosomal dominant inherited form of GTPCH deficiency (Segawa's disease or dopa-responsive dystonia (DRD) (Segawa et al., 1976, Ichinose et al., 1994)) mutations either in one of the three genes encoding for the biosynthetic enzymes (GTPCH, gene *GCH1*, PTPS, gene *PTS* and SR, gene *SPR*) or in one of the two genes encoding for the regenerating enzymes (PCD/DCoH, gene *PCBD* and DHPR, gene *QDPR*) (Thöny and Blau, 2006) (Figure 2).

In most cases, BH_4 deficiencies are affecting the biosynthesis of catecholamines (e.g. dopamine, norepinephrine and epinephrine) and indoleamines (serotonin and melatonin) (Nagatsu and Ichinose, 1999), the phenylalanine homeostasis (except for the dominant form of GTPCH deficiency and SR deficiency (Blau et al., 2001) as well as the NO production (Schmidt and Alp, 2007) (Campeau et al., 2007).

Symptoms associated with BH_4 deficiencies are characterized by developmental delay, progressive neurological deterioration, hypokinesia (abnormally diminished motor activity), hypersalivation and drooling, swallowing difficulties, truncal hypotonia (floppiness), increased limb tone, myoclonus (sudden twitching of muscles) and temperature instability (Bonafe et al., 2001, Thöny and Blau, 2006).

The diagnosis of BH_4 deficiencies is an integral part of the newborn screening program for phenylkenonuria (PKU) by Guthrie test (Guthrie and Susi, 1963) (in healthy normal people, phenylalanine levels are usually under 120 μmol/L) (Blau et al., 1996).

The definitive diagnosis of BH_4 deficiencies in newborns with blood phenylalanine levels greater then 120 μmol/L and in order children with consistent neurological symptoms, is achieved by urinary pterin analysis, DHPR activity measurements (e.g. in erythrocytes), BH_4 loading tests and examination of the cerebrospinal fluid (CSF) as well as DNA analysis (Blau et al., 1996, Longo, 2009). Moreover, the analysis of neopterin and biopterin as well as the neurotransmitter metabolites, HVA and 5-HIAA in the CSF, enables differentiation between severe and mild forms of BH_4 deficiencies (Blau et al., 1996).

Two BH_4 deficiencies occur without HPA namely the dominant form of GTPCH deficiency as well as SR deficiency and thus can not be detected by the newborn screening program for PKU (Blau et al., 2001, Thöny and Blau, 2006). Instead, these deficiencies are recognized either by typical clinical signs and symptoms or by analysis of the neurotransmitter metabolites and pterins in CSF and investigation of cultured skin fibroblasts (Blau et al., 2001).

1.5.1 PTPS deficiency

According to the international database of BH_4 deficiencies BIODEF (Blau et al., 1996) (available at www.biopku.org), the most common cause of BH_4 deficiencies is due to autosomal recessively inherited mutations in the *PTS* gene (approximately 54 % of all cases

of BH_4 deficiencies) (Figure 2). Among the 356 patients with PTPS deficiency (BIODEF), more than 52 mutant alleles have been found (International database of mutations causing tetrahydrobiopterin deficiencies, BIOMDB (available at www.bh4.org)).

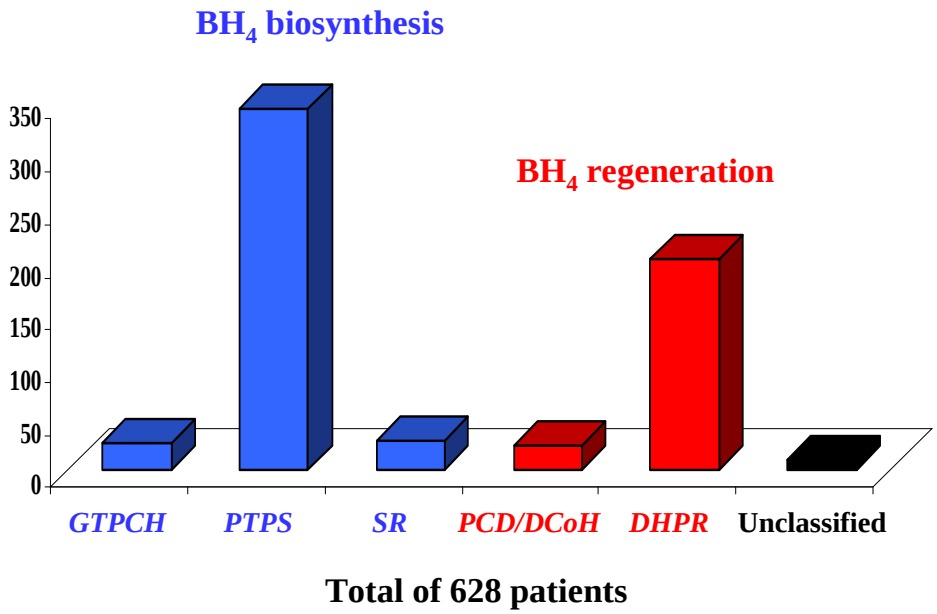

Figure 2: The most common cause of BH_4 deficiency is due to mutations in the *PTS* gene

Human BH_4 deficiency is caused by mutation in one of the three enzymes involved in the biosynthesis of BH_4 (GTPCH, PTPS and SR) or in one of the two enzymes involved in the recycling of BH_4 (PCD and DHPR). The most common cause of BH_4 deficiency is according to the international database of BH_4 deficiencies BIODEF (available at www.bh4.org) due to insufficient PTPS activity. Note that the *GCHFR* gene, encoding GFRP, is the only gene in the BH_4 metabolism for which no human deficiency has been described. The numbers of BH_4 deficient patients (total of 628) were obtained from the international database of BH_4 deficiencies BIODEF at www.bh4.org.

Patients with insufficient PTPS enzyme activity have difficulties with the conversion of NH_2TP to PPH_4, consequently this leads to an accumulation of NH_2TP. In the tissue, NH_2TP is dephosphoylated by various endogenous phosphatases to the intracellular active 7,8-dihydroneopterin which is predominant in several body fluids and can among others be detected in the urine and CSF as neopterin which is the oxidized form of 7,8-dihydroneopterin (1988). Moreover, PTPS-deficient patients develop HPA and neurological symptoms mainly due to lack of catecholamines and serotonin (Demos et al., 2005).

Mutations in the PTPS gene, which are distributed throughout all six exons (Thony and Blau, 1997), are associated with a broad spectrum of phenotypes ranging from HPA only to abnormal monoamine neurotransmitter biosynthesis.

In 80 % of the PTPS-deficient patients a severe central phenotype is observed with monoamine neurotransmitter deficiency assessed by CSF measurement of catecholeamines and serotonin metabolites (HVA and 5-HIAA) (Thöny and Blau, 2006, Longo, 2009). Conversely, 13 % of the PTPS patients have a mild peripheral phenotype characterized with moderate or transient HPA only (no neurological involvement and normal neurotransmitter homeostasis in the CSF) (Thöny and Blau, 2006, Longo, 2009). The molecular mechanisms for these phenotypic distinctions are unknown.

The patients with the severe/central form of PTPS deficiency require a combined therapy with oral neurotransmitter precursors (L-dopa, 5-hydroxytryptophan and carbidopa (AADC inhibitor)) as well as BH_4 supplementation whereas the patients with the mild/peripheral require "only" mono-therapy with BH_4 supplementation to maintain normal blood phenylalanine concentrations (Dudesek et al., 2001). Unfortunately, some infants do not respond to conventional therapy despite early diagnose and some of them died (Dudesek et al., 2001).

The severe form of PTPS deficiency resemble Parkinson's Disease (indicating a lack of dopamine in the basal ganglia) and is associated with truncal hypotonia, increased limb tone, postural instability, hypokinesia, choreatic or dystonic limb movements, gait difficulties, seizures (myoclonic and tonic clonic), hypersalivation and drooling due to swallowing difficulties as well as oculogyric crises (a paroxysm in which the eyes are held in a fixed position) (Dudesek et al., 2001, Blau et al., 1996). Moreover, ataxia (failure of muscular coordination), hyperreflexia (exaggeration of reflexes), hypothermia (body temperature below 35°C) as well as episodes of hyperthermia (in the absence of infections), drowsiness (sleepiness), irritability, lethargy (state of sluggishness, inactivity, and apathy), disturbed sleep pattern, ptosis (dropping upper eyelid), pinpoint pupils and convulsions (involuntary contractions) are often observed (Dudesek et al., 2001, Blau et al., 1996).

The symptoms may become evident in the first weeks of life (abnormal signs in the neonatal period includes poor sucking, impaired tone and microcephaly (abnormal smallness of the head)) but are mostly seen at an average age of 4 months (Dudesek et al., 2001).

In order to investigate the phenotypic distinctions associated with mutations in the PTS gene and to analyze the consequences of BH_4 deficiency and its potential treatment in more details, corresponding animal models are required.

1.6 Mouse models in the BH_4 metabolism

Since the development of transgenic mouse model technology, mouse models have become the leading mammalian model organism for studying human genetic diseases. Today, numerous mutated mouse lines exist, in fact estimations have suggested that approximately half of all the mouse genes have been targeted and corresponding mouse lines established (Beckers et al., 2009).

Several mouse gene knock-out programs such as the NIH knock-out mouse project (KOMP), the European Conditional Mouse Mutagenesis Program (EUCOMM), the North American Conditional Mouse Mutagenesis Project (NorCOMM) and the Texas A&M Institute for Genomic Medicine (TIGM) have all been established exclusively with the aim of generating a null mutation in every gene in the mouse genome (Thöny and Finnell, 2009).

Several mouse models for human defects in the BH_4 metabolism have been generated including the autosomal dominant from of GTPCH (DRD, Segawa's disease) (*hph-1* mouse as a model for dominantly inherited GTPCH deficiency), PTPS, SR, PCD/DCoH and DHPR ((Thöny and Gibson, 2006), (Thöny and Finnell, 2009) and (Werner et al., 2011)) (Table 1). So far no animal models for either the autosomal recessive form of GTPCH or GFRP have been generated (Table 1).

Table 1: Available mouse models within the BH_4 metabolism

Human BH_4 deficiency	**Available mouse model**	**References**
GTPCH, recessive	Not available	-
GTPCH, dominant (DRD)	Available (*hph-1*)	(Hyland et al., 2003, Sato et al., 2008, McDonald et al., 1988)
PTPS	Available (*Pts*-knock-out)	(Elzaouk et al., 2003, Sumi-Ichinose et al., 2001)
SR	Available (*Spr*-knock-out)	(Takazawa et al., 2008, Yang et al., 2006)
PCD/DCoH	Available (*DCoH*-knock-out)	(Bayle et al., 2002)
DHPR	Available (*Dpr*-knock-out)	Unpublished[a]
GFRP	Not available	-

Table 1 is adapted from (Thöny and Gibson, 2006), (Thöny and Finnell, 2009) and (Werner et al., 2011).

[a] Oral Presentation (BH4 Metabolism in the Brains of KO mice) at the 14th International Symposium on Pteridines and Folates by Prof. Hiroshi Ichinose.

The first BH_4-deficient mouse to be generated was a *Pts*-knock-out mouse (*Pts*-ko) with a complete disruption of the *Pts* gene (Ichinose et al., 2008).

Studies have shown that the homozygous *Pts*-ko mice died within hours after birth (Elzaouk et al., 2003, Sumi-Ichinose et al., 2001). The biochemical analysis revealed HPA, no PTPS enzyme activity in liver and brain, high levels of neopterin in liver and brain, low levels of biopterin in liver and brain as well as neurotransmitter deficiency in the CNS due to undetectable or very low levels of dopamine and serotonin (Elzaouk et al., 2003). Nevertheless, daily oral administration of BH_4 and neurotransmitter precursors (5-hydroxytryptophan and L-dopa) rescued the mice from lethality. Yet, the overall condition of the homozygous *Pts*-ko mice was very serious partly because of severe dwarfism due to growth deficiency (Elzaouk et al., 2003).

Patients with PTPS deficiency do not reveal newborn lethality although symptoms may be notable during the neonatal period (Elzaouk et al., 2003). Furthermore, low birth weight and microcephaly, which are typical for human PTPS deficiency, were not observed in the homozygous *Pts*-ko mice (Elzaouk et al., 2003). On the other hand, some symptoms like hypersalivation and temperature instability were found both in the *Pts*-ko mice and in the human patients (Elzaouk et al., 2003).

Mouse models for SR and PCD/DCoH deficiencies have all been successful generated although these models were only partially reflecting the corresponding human counterparts (Takazawa et al., 2008, Yang et al., 2006, Bayle et al., 2002). As an example, the *Spr* knock-out (*Spr*-ko) mice exhibit HPA and severe growth retardation (Yang et al., 2006, Takazawa et al., 2008, Ichinose et al., 2008).

1.6.1 German Mouse Clinic (GMC)

To take full advantage of transgenic mice as model organism for human diseases systematic (assessing all mouse lines) and systemic (assessing all organs) phenotypings are crucial (Beckers et al., 2009).

Comprehensive systemic phenotyping at the German Mouse Clinic (GMC) in Munich has thus far identified new and unexpected phenotypes in 96 % of the more then 100 mutant mouse lines investigated (Gailus-Durner et al., 2005, Fuchs et al., 2009, Beckers et al., 2009, Fuchs et al., 2011).

A complete primary screen at the GMC requires 80 mice (20 mutant males, 20 mutant females, 20 male controls and 20 female controls). The 80 mice which are born with 7 days

difference to reduce the heterogeneity within the cohort are imported to the clinic at the age of 8 weeks. After adaptation, the primary screening is started in the second week and completed after 10 weeks when the mice are at the age of 18 weeks (Figure 3).

Within the primary screen, the mice are distributed into two different pipelines; each pipeline consists of a cohort of 10 animals per sex and genotype.

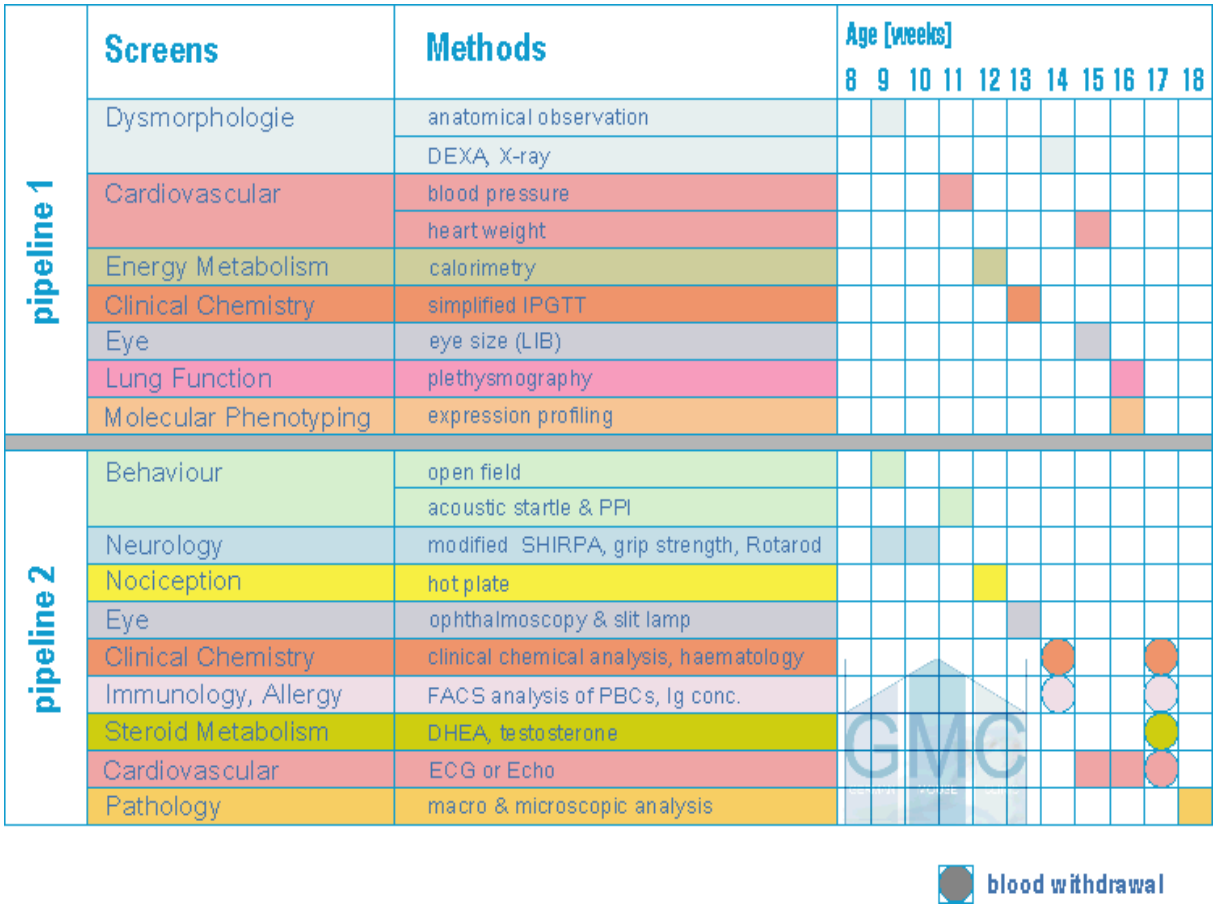

Figure 3: Schematic representation of the primary screening at the German Mouse Clinic (GMC)

The primary screening at the GMC consists of 550 parameters and detailed pathological examination. Within the primary screening), the 80 mice (20 mutant males, 20 mutant females, 20 male controls and 20 female controls) are distributed into two different pipelines; each pipeline consists of a cohort of 10 animals per sex and genotype. The primary screening takes 10 weeks and ends when the mice are at the age of 18 weeks. Based on the out-come of the primary screening, decisions for secondary and tertiary screening are made. DEXA: Dual-energy X-ray absorption, IPTGTT: Intraperitoneal glucose tolerance test, LIB: Laser interference biometry, PPI: Pre-pulse inhibition, SHIRPA: a protocol for comprehensive behaviour assessment, FACS: fluorescence activated cell sorting, PBC: peripheral blood cell, Ig: Immunoglobulin, DHEA: Dehydroepiandrosteron, ECG: electrocardiogram.

Figure from http://www.mouseclinic.de/index.php?id=11119.

In the first pipeline, the mice are subjected to non-invasive tests which among others investigate the dysmorphology, blood pressure, calorimetry, intraperitoneal glucose tolerance, bone densitometry, axial eye length and lung function (Fuchs et al., 2009) (Figure 3).

In the second pipeline, the mice are subjected to a series of behavioural and neurological tests including open field, modified SHIPRA, grip strength, rotarod, acoustic startle, pre-pulse inhibition, hot plate test as well as examination of the eyes and vision (Fuchs et al., 2009). Furthermore, blood-samples are taken to examine different parameters within immunology, allergy, clinical chemistry, cardiovascular and steroid parameters (Fuchs et al., 2009) (Figure 3).

Depending on the primary screening, secondary and tertiary screenings are performed conversely these additional screenings requires a new cohort of mice (Beckers et al., 2009, Fuchs et al., 2009).

1.7 Metabolism and function of serotonin

Serotonin which is also known as 5-hydroxytryptamine or 5-HT is synthesized from the essential amino acid tryptophan (Trp) via 5-hydroxytryptophan (5-OH-Trp) by the action of two enzymes: BH_4-cofactor-dependent TPH and AADC, respectively (Haavik et al., 2008). TPH exist in two isoforms with distinct function and localization, a peripheral isoform called TPH1 and a neuronal isoform called TPH2 (Walther and Bader, 2003, Haavik et al., 2008). Degradation of serotonin to its end-metabolites 5-hydroxyindolacetic acid (5-HIAA) involves monoamine oxidase A (MAO-A) in combination with aldehyde dehydrogenase (ALDH) (Figure 1) (Haavik et al., 2008).

In the periphery, the primary source for serotonin is in the gut, where it is synthesized by enterochromaffin cells of the mucosa. The serotonin production in the intestinal wall makes up more than 90% of the total generated serotonin. From the enterochromaffin cells, the serotonin is releases into the bloodstream, where it is taken up by platelets and transported to all vascularised tissues (Haavik et al., 2008, Alenina et al., 2006).

Serotonin is involved in a broad spectrum of biological, behavioural and psychological conditions including mood, sleep, depression, autism, memory, learning, appetite, eating disorders, obesity, anxiety, seizures, aggression, migraine, alcoholism, cognition, attention-deficit/hyperactivity disorder (ADHD), body temperature, obsessive-compulsive disorders (OCD), sexual and motor activity, schizophrenia, pain perception, cardiovascular function,

muscle contraction homeostasis, endocrine functions, adult respiratory distress syndrome (ARDS), substance abuse disorders, neurodegenerative disorders, as well as social interaction and affiliation (Hahn and Blakely, 2007, Murphy et al., 2008, Haavik et al., 2008).

1.7.1 Serotonin and brain development

In addition to its neurotransmitter function in the mature nervous system, serotonin also plays an important role during the development of the brain. In the human brain, serotonergic neurons are first evident by 5 weeks of gestation which makes serotonin the first of the monoamine neurotransmitter systems to be present in the terminal regions (Whitaker-Azmitia, 2001). This fact emphasizes the importance of serotonin during brain development. The amount of serotonergic neurons increases rapidly through the first 10^{th} week of gestation and by the 15^{th} week of gestation, the typical organization of serotonin cell bodies into the raphe nuclei can be seen. The serotonin levels then increase slowly throughout the first two years of life and then decline to the adult levels by the age of 5 years (Whitaker-Azmitia, 2001).

Serotonin indirectly stimulates and inhibits its own growth thereby insuring that the serotonin terminals reach their correct target areas (Whitaker-Azmitia, 2001).

At the target areas serotonin influences several processes such as neurogenesis and/or neuronal removal, dendritic refinement, synaptic remodelling and maintenance and cell migration (Whitaker-Azmitia, 2001). Studies on rats have shown that removal of serotonin during very early fetal development causes a permanent reduction in the number of neurons in adult brain (Whitaker-Azmitia, 2001). The concentration of serotonin can be changed during development by viral infection, malnutrition, social enrichment and/or isolation, hypoxia and stress (Whitaker-Azmitia, 2001).

1.7.2 Neurohormone

Beside, numerous roles as a monoamine neurotransmitter both in the central and peripheral nervous system, serotonin is also a neurohormone that via the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-spinal-adrenal (HAS) axis modulates the glucocorticoid secretion upon response to stressful stimuli (Mueller et al., 2010, Lowry, 2002). Subsequently, the released glucocorticoids influence metabolic, immune and developmental processes (Mueller et al., 2010). Studies on newborns have linked promoter region polymorphisms within the serotonin transporter gene to an increased stress reactivity of the HPA-axis (Mueller et al., 2010).

1.8 Serotonin transporters (SERT and PMAT)

1.8.1 Serotonin re-uptake transporter (SERT)

The human serotonin re-uptake transporter (SERT or 5-HTT) which is encoded by the *SLC6A4* gene belongs together with the genes encoding for dopamine transporters (DAT, *SLC6A3*) and norepinephrine transporters (NET, *SLC6A2*) to the solute carrier 6A (*SLC6A*) family, (member 4) (Hahn and Blakely, 2007, Torres et al., 2003). Solute carrier 6A (*SLC6A*) is a gene family of ion-coupled plasma membrane co-transporters that mediate the movement of their substrate (e.g. serotonin) into cells to regulate synaptic transmission, neurotransmitter recycling, metabolic function and fluid homeostasis (Hahn and Blakely, 2007).

The *SLC6A4* gene is located on chromosome 17q11.1-17q12 and consists of 15 exons spanning approximately 40 kb (Murphy et al., 2008, Ramamoorthy et al., 1993). Transcription of the *SLC6A4* gene leads to a plasma membrane protein composed of 630 amino acids, 12 transmembrane domains as well as cytoplasmic amino and carboxy termini (Torres et al., 2003). The transcriptional activity of the *SLC6A4* gene is modulated by several factors among these are two common variable number of tandem repeat (VNTR) polymorphisms in the 5-HTT gene-linked polymorphic promoter (5-HTTLPR) region and in intron 2 (serotonin transporter intron 2 VNTR or STin2 VNTR), respectively.

The 5-HTTLPR promoter sequence which is located about 1 kb upstream of the transcription start site contains two VNTR polymorphisms known as the long (L) or short variant (S), which differ with respect to an insertion/deletion of 44 base-pairs (Esterling et al., 1998, Heils et al., 1996, Lesch et al., 1996). The long 5-HTTLPR variant with 16-repeats and short 5-HTTLPR variant with 14-repeats are known to lead to different expressions of the serotonin re-uptake transporter protein in pre-synaptic nerve membranes (Heils et al., 1996, Lesch et al., 1996, D'Souza and Craig, 2006).
Studies have shown that the long 5-HTTLPR variant expresses significantly more SERT mRNA and subsequently more SERT protein on the pre-synaptic nerve membrane compared to the L/S and S/S variants (Heils et al., 1996, D'Souza and Craig, 2006) (Figure 4).
As a consequence, the L/L 5-HTTLPR variant leads to a lowered concentration of serotonin in the synaptic cleft as the serotonin re-uptake is more than 2-fold higher in L/L expressing cells than cells carrying one (L/S) or two (S/S) copies of the short 5-HTTLPR variant of the promoter (Lesch and Gutknecht, 2005) (Figure 4).

Opposite, the short variant of 5-HTTLPR leads to a reduced rate of SERT mRNA transcription thus less SERT protein is expressed on the pre-synaptic nerve membrane and the serotonin re-uptake activity is lowered (Lesch et al., 1996, D'Souza and Craig, 2006) (Figure 4).

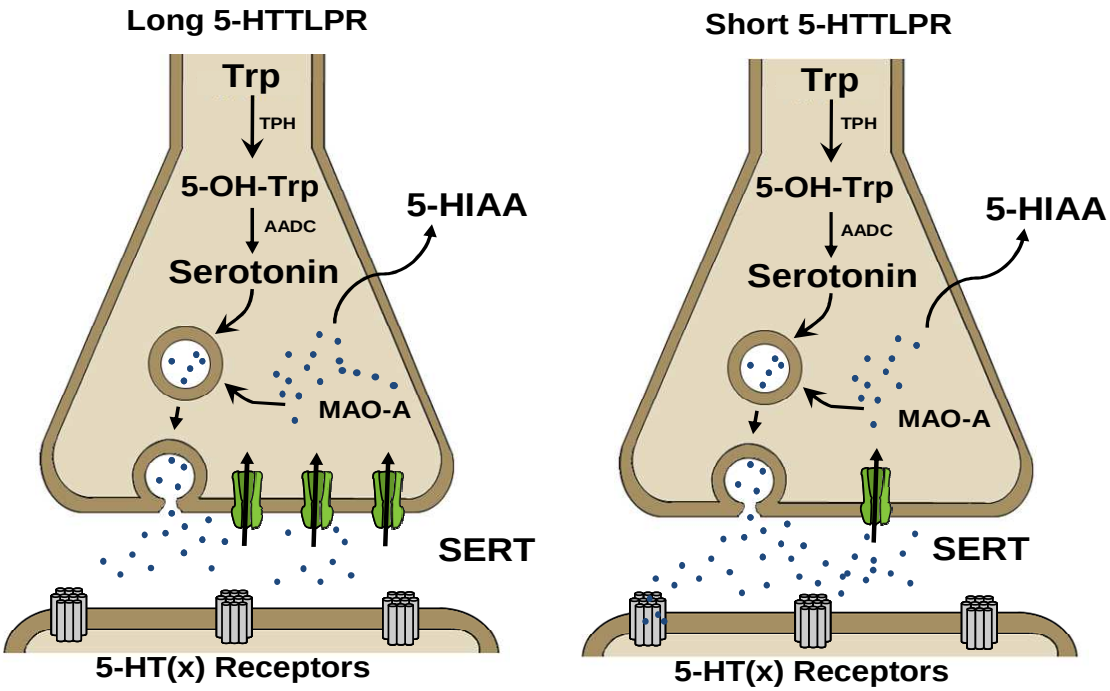

Figure 4: Expression of the long 5-HTTLPR and short 5-HTTLPR in the pre-synaptic nerve terminal
In the pre-synaptic nerve terminal, serotonin is synthesized from tryptophan (Trp) via 5-hydroxytryptophan (5-OH-Trp) by the BH_4-dependent tryptophan hydroxylase (TPH) and the aromatic amino acid decarboxylase (AADC). Serotonin is released from the pre-synaptic axon terminal into the synaptic cleft by serotonin-carrying vesicles. The action of serotonin in the synaptic cleft is tightly regulated by serotonin re-uptake transporter (SERT) located in the pre-synaptic plasma membrane. The long 5-HTTLPR variant of the *SLC6A4* gene produces significantly more SERT mRNA and protein than the short 5-HTTLPR variant. Consequently, the long 5-HTTLPR variant leads to lower concentration of the serotonin in the synaptic cleft compared to the short 5-HTTLPR variant. After re-uptake of serotonin into the pre-synaptic nerve terminal by SERT, serotonin can either be recycled (back into the serotonin-carrying vesicles by vesicular monoamine transporter (VMAT)) or degraded to its end-metabolite 5-hydroxyindoleacetic acid (5-HIAA) by monoamine oxidase A (MAO-A). Figure adapted from (Canli and Lesch, 2007) and (Gerretsen and Pollock, 2008).

Another common variant of the *SLC6A4* locus, which potentially modulates the serotonin re-uptake transport protein in terms of function and expression, is a variable number of tandem repeat (VNTR) polymorphism of 17-bp repeats in intron 2, STin2.9, STin2.10, and STin2.12, containing 9, 10 and 12 copies of the VNTR element, respectively (MacKenzie and Quinn, 1999).

Expression in transgenic embryos and embryonic stem cells suggests that the STin2 VNTR polymorphism acts as a regulator of transcription (Hahn and Blakely, 2007). The STin2.12 allele, which is the most common of the three VNTRs, has been reported to cause a significantly higher expression of the *SLC6A4* gene in embryonic mouse rostral hindbrain compared to the STin2.9 and STin2.10 alleles demonstrating that STin2.12 is a transcriptional enhancer (MacKenzie and Quinn, 1999). In contrast, a smaller number of VNTRs such as STin2.9 and STin2.10 have been hypothesized to influence the stability of the transcription complexes and steady-state concentrations of mRNA, thereby causing less *SLC6A4* gene expression than STin2.10 (Ogilvie et al., 1996).

Besides, the non-coding functional VNTR polymorphisms of the *SLC6A4* gene in the promoter region and intron 2, a number of functional gene variants in the coding alleles have also been shown to modulate the SERT activity in terms of gene expression, protein trafficking or protein regulation (Lesch et al., 1996, Murphy et al., 2008, Prasad et al., 2009).

Among them is the non-synonymous variant Gly56Ala in exon 3, which has been reported to be associated with autism and rigid compulsive behaviours (Sutcliffe et al., 2005).

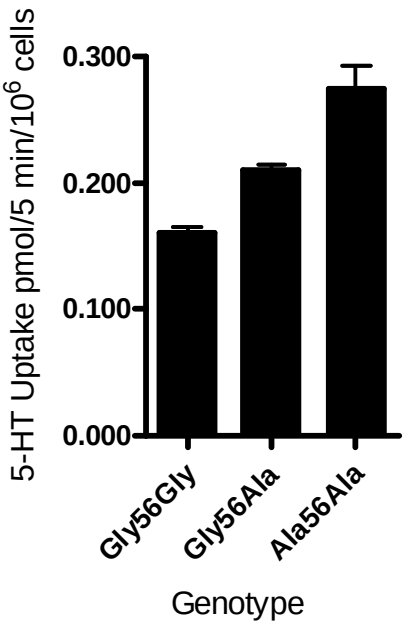

Figure 5: Gly56Ala in SERT causes an up-regulation regarding serotonin transport (gain of function)
Both the heterozygous (Gly56Ala) and homozygous (Ala56Ala) amino acid substitution at position 56 in the SERT protein have been shown to cause a significant increase in serotonin uptake (5-HT Uptake pmol/5min/10^6 cells) when transfected into lymphoblastoid cells compared to cells expressing the wild-type (Gly56Gly) SERT protein. This figure is adapted from (Sutcliffe et al., 2005).

This alanine to glycine exchange at position 56, which showed constitutively elevated SERT activity, is located in the N-terminal intracellular tail of the SERT protein (Sutcliffe et al., 2005) (Figure 6). Pharmacological studies of serotonin re-uptake by lymphoblastoid cells expressing the heterozygous Gly56Ala or homozygous 56Ala protein mutants indicated a progressive gain of function by 1.4 or 1.8 fold, respectively, despite normal surface expression compared to lymphoblastoid cells expressing the wild-type SERT (Gly56) (Sutcliffe et al., 2005, Veenstra-Vanderweele et al., 2009) (Figure 5).

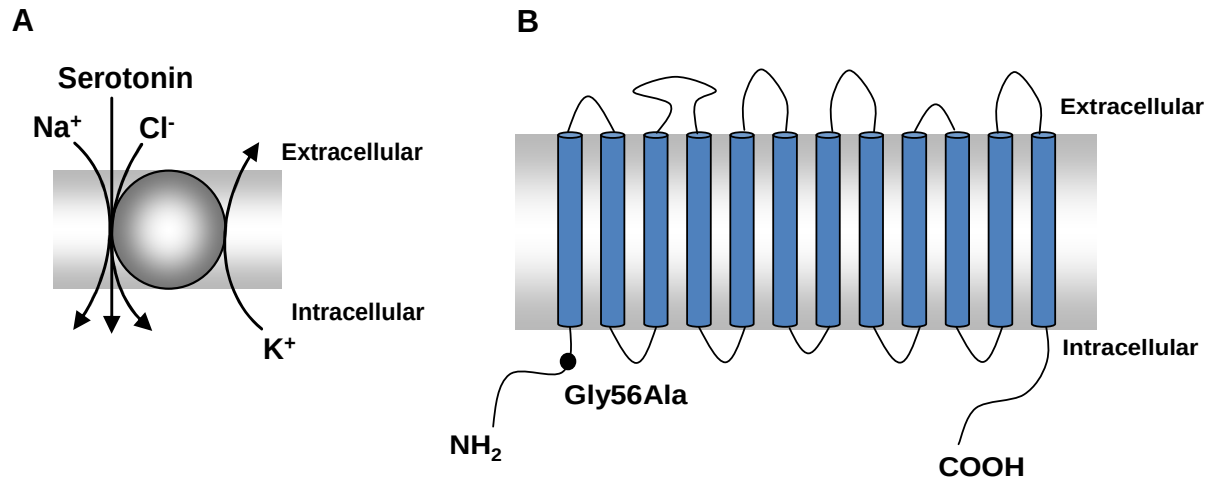

Figure 6: Ion coupling stoichiometry for SERT and location of SERT-coding variant Gly56Ala

A: External serotonin is transported together with Na^+ and Cl^- ions into the intracellular space by SERT (grey circle). This co-transport of serotonin, Na^+ and Cl^- ions causes a conformational change within the SERT protein which allows an intracellular K^+ ion to be transported outwards, which makes the transport cycle electronneutral and the SERT protein capable of binding another molecule of external serotonin. This figure is adapted from (Rudnick, 1998). B: Schematic model representation of the 12 transmembrane SERT with intracellular NH_2 and COOH termini. The functional SERT-coding variant Gly56Ala (black circle), which has demonstrated elevated SERT activity, is located in the NH_2-terminal intracellular tail of the SERT protein. This figure is adapted from (Prasad et al., 2009), (Veenstra-Vanderweele et al., 2009) and (Sutcliffe et al., 2005).

The driving force for the re-uptake of serotonin by SERT is the ion concentration gradient of Na^+ and Cl^- ions generated by the plasma membrane ion-pumping Na^+/K^+ ATPase (Torres et al., 2003). The binding of serotonin together with Na^+ and Cl^- ions (one of each) to the extracellular part of SERT causes a conformational change allowing the concomitant translocation of serotonin, Na^+ and Cl^- ions into the cytoplasm as well as exposure of an intracellular K^+ ion binding site (Figure 6).

In exchange, one intracellular K^+ ion is transported outwards, which makes the transport cycle electronneutral and the SERT protein capable of binding another molecule of external serotonin (Torres et al., 2003, Barker et al., 1999, Rudnick, 1998) (Figure 6).

In the brain, monoamine transporters are expressed almost exclusively in the neurons that contain their cognate transmitter which means that SERT is expressed in the median and dorsal raphe nuclei (Torres et al., 2003). Additionally, SERT protein has also been found in peripheral locations, including the platelets, intestinal tract, placenta, mast cells, basophils and adrenal gland (Torres et al., 2003). Altogether, demonstrating that SERT serves many different physiological functions both in the CNS and the periphery (Rudnick, 1998).

The SERT protein is a target for several pharmacological agents that affect brain function, including psychostimulants (cocaine and amphetamines), antidepressants and neurotoxins (Torres et al., 2003). The pharmaclolgical agents, which bind to SERT and inhibit the serotonin re-uptake transport can either act as substrates, selective serotonin re-uptake inhibitors (SSRIs) or non-selective serotonin re-uptake inhibitors (SRIs) (Torres et al., 2003). Both the selective and non-selective serotonin inhibitors are thought to occupy sites overlapping the substrate binding site and are widely used in treatment of neuropsychiatric disorders including affective disorders, depression, anxiety disorders, obsessive-compulsive disorder (OCD), autism, eating disorders and substance abuse such as alcoholism (Lesch and Gutknecht, 2005, Murphy et al., 2004).

1.8.2 Plasma membrane monoamine transporter (PMAT)

The human plasma membrane monoamine transporter (PMAT or ENT4) which is encoded by the *SLC29A4* gene, belongs to the equilibrative nucleoside transporters (ENTs) family, SLC29 (member 4) (Baldwin et al., 2004). This family is known to be nucleoside transporters that specifically transport nucleoside and their structural analogs (Kong et al., 2004). However, characterization studies have shown that PMAT functions as a polyspecific organic cation transporter for substrates such as 1-methyl-4-phenylpyridinium (MPP^+), tetrahylammonium and biogenic amines including serotonin, dopamine, norepinephrine, epinephrine as well as histamine.

Unlike the rest of the SLC29 family, PMAT only minimally interacts with nucleosides, nucleoside analogs, nucleobases or nucleotides (Engel et al., 2004, Zhou et al., 2007). Hence, the substrate selectivity of PMAT is more similar to the organic cation transporters (OCTs) in

the SLC22 family then the substrate selectivity of the SLC29 family (Zhou et al., 2007). The deviation of *SLC29A4* from the rest of the SLC29 family is also reflected in the low homology (~ 20 %) (Engel et al., 2004). Despite similar substrate selectivity with OCTs its sequence identity to the OCTs is only 11-14 % (Zhou et al., 2007).

The *SLC29A4* gene is located at chromosome 7p22.1 and is translated into a plasma membrane transporter protein composed of 530 amino acids with a calculated molecular mass of 58 kDa (Engel et al., 2004) (Figure 24). Hydropathy analysis have predicted the presence of 11 membrane-spanning domains, a cytoplasmic N-terminus and an extracellular C-terminus (Engel et al., 2004) (Figure 24).

Whereas SERT-mediated transport is Na^+ and Cl^--dependent, the re-uptake activity of PMAT does not rely on the presence of Na^+ and Cl^- ions thus the PMAT-mediated re-uptake transport is ion-independent (Engel et al., 2004). Instead, the PMAT-mediated transport appears to be sensitive to changes in the membrane potential (Engel et al., 2004). Beside the ion-independent transport, PMAT also differentiate from SERT in terms of substrate affinity and inhibitor insensitivity.

The substrate affinity of PMAT for serotonin and dopamine are 2-3 orders of magnitude lower than those of SERT and DAT. However, the Vmax values of PMAT-mediated serotonin and dopamine transport are 2-3 of magnitude higher. Together, suggesting that PMAT in contrast to SERT is a low affinity but with high capacity transport for serotonin (Daws, 2009, Engel et al., 2004). Moreover, PMAT has shown to be insensitive towards the high affinity inhibitors of SERT such as fluoxetine (Prozac) and tricyclic antidepressant desipramine (Engel et al., 2004, Vialou et al., 2007).

Northern blot analysis of human PMAT mRNA transcript has shown that PMAT is widely expressed in the CNS including the cerebellum, cerebral cortex, medulla, spinal cord, occipital pole, frontal lobe, temporal lobe and putamen (Engel et al., 2004). Beside expression of PMAT in different brain tissue, PMAT has also been detected in various peripheral tissue including the skeletal muscles, kidney, heart and liver (Engel et al., 2004).

1.8.3 *SLC6A4* gene variants and neurological behaviour, traits and disorders

Several neuropsychiatric conditions have been associated with SERT gene variations, among these are bipolar affective disorder, depression, anxiety, OCD, suicide, eating disorders, schizophrenia, substance-abuse disorders such as alcohol abuse, autism, ADHD and neurogenerative disorders (Hahn and Blakely, 2007, Lesch and Gutknecht, 2005).

In addition, SERT gene variations are also implicated in diseases of peripheral systems particular myocardial infarction, primary pulmonary hypertension, irritable bowel syndrome and sudden infant death syndrome (SIDS) have also been linked to *SLC6A4* gene (Hahn and Blakely, 2007, Murphy et al., 2008, Murphy et al., 2004).

Numerous association studies have linked variants within the *SLC6A4* gene with autism both in terms of the short and long 5-HTTLPR allele, STin2 VNTRs and coding variants in SERT such as Gly56Ala in exon 3 (Cook et al., 1997, Klauck et al., 1997, Sutcliffe et al., 2005, Prasad et al., 2009). Subsequently, studies have yielded inconsistent findings (Veenstra-Vanderweele et al., 2009, Huang and Santangelo, 2008, Sakurai et al., 2008).

The *SLC6A4* gene is of special interest for autism for at least three reasons (Huang and Santangelo, 2008):

1) Serotonin has been shown to regulate the development of the CNS (Whitaker-Azmitia, 2001) and to be involved in a broad spectrum of behavioural and psychological processes (e.g. social behaviour, aggression and anxiety) and various psychiatric disorders (Murphy et al., 2004).

2) Several studies have reported that approximately one-third of all autistic patients have elevated levels of whole blood (or platelet) serotonin (hyperserotoninemia) (Cross et al., 2008, Leboyer et al., 1999). This hyperserotoninemia which is believed to be caused by abnormal maturation of the serotonin system (Leboyer et al., 1999, Whitaker-Azmitia, 2001), is also suggested to be either directly or indirectly responsible for the immune abnormalities observed among autistic subjects (Burgess et al., 2006). In addition, alterations of serotonin synthesis in the dentatothalamocortical pathway were found in autistic boys (Chugani et al., 1997, Chugani, 2002). Altogether, suggesting that abnormal serotonergic transmission may be important in the pathogenesis of autism (Chugani et al., 1997, Chugani, 2002) .

3) Increased repetitive behaviours and irritability were observed in autistic patients when subjected to a dietary depletion of the serotonin precursor tryptophan due to an expected reduction in extracellular serotonin availability (Veenstra-Vanderweele et al., 2009). Opposite, increased extracellular serotonin availability due to administration of SSRIs to individuals with autism led to reduced symptoms of irritability and rigid-compulsive behavior (Veenstra-Vanderweele et al., 2009, Buitelaar and Willemsen-Swinkels, 2000).

1.9 Autism Spectrum Disorder (ASD)

Autism was first recognized in 1943 by Leo Kanner in 11 boys with autistic disturbances of affective contact (Kanner, 1943). Throughout, the 1960s and 1970s autism was considered to be a form of psychosis or childhood schizophrenia. Additionally, the conventional perception was that autism was related to parenting styles (Geschwind, 2009). In the later decades, it was demonstrated that autism is not caused by bad parenting but rather due to heritable factors determined by multiple genes whose identity or variation may be distinct in different families (Spence, 2004, Prasad et al., 2009). Moreover, autism was found not to be a mental illness but instead a complex lifelong neuro-developmental disorder affecting the functioning of the brain and which typically appears during the first three years of life (Lamb et al., 2009).

Classical autism, which is also known as Kanner autism or typical autism belongs together with Asperger's syndrome and pervasive developmental disorder – not otherwise specified (PDD-NOS) (or atypical autism) to autism spectrum disorders (ADSs) (Levy et al., 2009).

According to the *Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition, (DSM-IV),* the diagnostic criteria for ASDs are based on severe and pervasive impairments within three behavioural domains 1) social interaction, 2) language, verbal and non-verbal communication as well as imaginative play and 3) repetitive, restrictive, stereotyped or unusual patterns of behaviours, interests and activities (Muhle et al., 2004, Geschwind, 2009). The diagnostic assessment of ASDs depends on the number and distribution of endorsed behavioural descriptions in each of the three domains as well as on the age of onset (Muhle et al., 2004). In addition, to the three domains, several other areas of clinical dysfunction are observed in autistic patients including sensory abnormalities, motor abnormalities, sleep disturbance, gastrointestinal symptoms and epilepsy (Geschwind, 2009).

Strong evidence has reported that ASDs are highly heritable disorders. The heritability, which has been estimated from family and twin studies, suggests that about 90 % of variance is attributable to genetic factors (Levy et al., 2009). ASDs are due to multi-factors which act together to produce the phenotype (i.e. gene-gene and/or gene-environmental interactions) (Levy et al., 2009). Despite, the evidence from twin and family studies, the identity and number of genes involved are not yet known (Muhle et al., 2004). Current models estimate that at least 20 genes interact with each other and probably with environmental factors to create these complex disorders (Spence, 2004).

1.10 Aim of the thesis

Transgenic mouse models are essential for studying gene function and provide an important tool in experimental medicine and in modelling human genetic disorders. This also applies to human BH_4 deficiencies, which are a highly heterogeneous group of rare autosomal recessive inherited disorders, mostly characterized by HPA, reduced BH_4 levels and monoamine neurotransmitter deficiency in the CNS. For a better understanding of BH_4 deficiencies, the need for efficient animal models to study various aspects of human BH_4 deficiency is crucial.

In the design phase of this thesis in 2007, several mouse models for human defects in the BH_4 metabolism had already been generated including dominant form of GTPCH, PTPS, SR and PCD/DCoH. The first mouse model for PTPS, a complete knock-out of the *Pts* gene (*Pts*-ko) exhibited newborn lethality and revealed severe conditions consequently the *Pts*-ko mouse was not a suitable mouse model to study BH_4 deficiency.

This motivated us to generate a new *Pts* mouse models: a homozygous *Pts*-R15C-ki (*Pts*-ki/ki) and a compound heterozygous *Pts*-R15C-ki/*Pts*-ko (*Pts*-ki/ko) to study human BH_4 deficiency. We aimed to explore whether the *Pts*-ki/ki mice would result in a mild or peripheral phenotype and whether the *Pts*-ki/ko mice would generate a severe or central phenotype without newborn lethality, respectively as this potential could reveal the phenotypic diversities observed in human BH_4 deficiencies (Chapter 2).

The *GCHFR* gene is the only gene within the BH_4 metabolism for which no human deficiency or mouse model has been described. In consideration of this, we aimed to generate the first animal model for GFRP, a conditional knock-out for the murine *Gchfr* gene to study the *in vivo* regulatory role of GFRP in liver and brain (Chapter 3).

Furthermore, using a genetic candidate gene approach, we aimed to analyse a cohort of patients with ASDs and normal, unknown or isolated low 5-HIAA in the CSF. Due to the findings in the *SLC6A4* and *SLC29A4* genes coding for SERT and PMAT, respectively, we addressed by whole exome sequencing, the genetic characterization linking isolated low 5-HIAA in CSF, ASDs and serotonin re-uptake-transporter mutations (Chapters 4 and 5).

Taken together, the four studies presented in this dissertation aim to cover different aspects within 6-pyruvovyl-tetrahydropterin synthase (PTPS), GTP cyclohydrolase feedback regulatory protein (GFRP), serotonin re-uptake transporter (SERT) and plasma monoamine membrane transporter (PMAT) in order to get a better understanding of tetrahydrobiopterin and monoamine neurotransmitter deficiency in mouse models and human disease.

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2

New mouse models to study BH_4 deficiency by targeting the 6-pyruvoyl-tetrahydropterin-synthase (*Pts*) gene

Running title: New mouse models to study BH_4 deficiency

This part is a manuscript in preparation as Adamsen et al.

2 New mouse models to study BH_4 deficiency by targeting the 6-pyruvoyl-tetrahydropterin-synthase (*Pts*) gene

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2.1 Abstract

Human BH_4 deficiencies are a highly heterogeneous group of rare inherited neurological disorders, primarily due to recessive mutations in the biosynthetic 6-pyruvoyl-tetrahydropterin-synthase (*PTS*) gene. The majority of PTPS deficient patients exhibit a severe or central phenotype with brain monoamine neurotransmitter deficiency, whereas the minority has a mild or peripheral phenotype characterized with hyperphenylalaninemia (HPA) only. Previous studies have shown that the homozygous *Pts* knock-out mouse (*Pts*-ko) displayed newborn lethality. Therefore we established an alternative *Pts* mouse model by 'knocking-in' a single codon exchange in the *Pts* gene (*Pts*-ki). Homozygous *Pts*-R15C-ki mice (*Pts*-ki/ki) showed no signs of HPA or brain monoamine neurotransmitter deficiency, but significant reduced PTPS enzyme activity and elevated neopterin in liver and brain.

In addition, we bred compound heterozygous *Pts*-R15C-ki/*Pts*-ko (*Pts*-ki/ko) mice which gave rise to two phenotypes: one dying 3-4 days after birth "affected" *Pts*-ki/ko and one which survived "unaffected" *Pts*-ki/ko.

The "affected" *Pts*-ki/ko mice showed significant reduced PTPS enzyme activity and biopterin in liver and brain, elevated neopterin in liver and brain, mild HPA as well as monoamine neurotransmitter deficiency in the CNS. In comparison, "unaffected" *Pts*-ki/ko mice showed reduced PTPS activity in liver and brain but normal levels of blood phenylalanine. Furthermore, the biopterin and neopterin levels (both in liver and brain) as well as the brain monoamine neurotransmitters were found to be within the normal range.

In summary, the "affected" *Pts*-ki/ko mice have potential to be a suitable mouse model to study BH_4 deficiency as they mimic the biochemical hallmarks of human BH_4 deficiency

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2.2 Introduction

BH_4, which is generated from guanosine triphosphate (GTP) by three enzymes: GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) (Thöny et al., 2000) (Figure 1), is an essential cofactor for the three aromatic amino acid hydroxylases: phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylases 1/2 (TPH1, peripheral and TPH2, neuronal (Walther and Bader, 2003)). Furthermore, BH_4 is cofactor for all three isoforms of nitric oxide synthase (neuronal, inducible and endothelial NOS) as well as alkylglycerol monooxygenase (AGMO) (Wei et al., 2003, Watschinger et al., 2010, Werner et al., 2003) (Figure 1). As a consequence of the hydroxylation of the three aromatic amino acid hydroxylases, BH_4 is regenerated by the action of the two regenerating enzymes: pterin 4a-carbinolamine dehydratase (PCD/DCoH) and dihydropteridine reductase (DHPR) (Thöny et al., 2000) (Figure 1).

Human BH_4 deficiencies are caused by genetic defects in either one of the three genes encoding for the biosynthetic enzymes (GTPCH, PTPS and SR) or in one of the two genes encoding for the regenerating enzymes (PCD/DCoH and DHPR) (Thöny and Blau, 2006) (Figure 2). PTPS deficiency, which is the most common cause of BH4 deficiency, is associated with a broad spectrum of phenotypes range from severe and central to mild and peripheral. The molecular mechanisms for these phenotypic diversities are unknown.

The first BH_4-deficient mouse generated was a *Pts*-knock-out mouse (*Pts*-ko) with a complete disruption of the *Pts* gene (Elzaouk et al., 2003, Sumi-Ichinose et al., 2001). These studies have shown that the homozygous *Pts*-ko mice died within hours after birth, which indicated newborn lethality. The biochemical analysis revealed HPA (1290-1414 μmol/L), no PTPS enzyme activity, high levels of neopterin (liver and brain), low levels of biopterin (liver and brain) as well as neurotransmitter deficiency in the CNS due to undetectable or very low levels of dopamine and serotonin, but with normal NO values (Elzaouk et al., 2003). Despite, daily administration of BH_4 and neurotransmitter precursors which rescued the homozygous *Pts*-ko mice from newborn lethality, the homozygous *Pts*-ko mice were among others found to be sexually immature and to suffer from dwarfism due to severe growth deficiency (Elzaouk et al., 2003).

The overall very bad condition of the homozygous *Pts*-ko mice and the phenotypic diversities between the mouse ko-mutant and human patients motivated us to generate new *Pts* mouse models by ‘knocking-in’ a single codon exchange in the *Pts* gene (*Pts*-R15C-ki).

Besides generating a *Pts*-ki/ki mouse which was expected to reflect the mild or peripheral phenotype, we also generated a compound heterozygous *Pts*-R15C-ki/*Pts*-ko (*Pts*-ki/ko) mouse which was expected to reflect the severe or central phenotype of PTPS deficiency but without newborn lethality. The compound heterozygous mouse was generated by breeding the *Pts*-R15C-ki allele into the *Pts*-ko allele.

In this study, we successfully generated *Pts*-ki/ki and *Pts*-ki/ko mice. Moreover, we found that *Pts*-ki/ki mice are associated with a very mild phenotype that biochemically is characterized by reduced PTPS enzyme activity (liver and brain) as well as increased neopterin (liver and brain) and that they exhibit an abnormal fat distribution when subjected to standard mouse chow or high fat diet for 10 weeks. Furthermore, we found that “affected” *Pts*-ki/ko mice, in contrast to *Pts*-ki/ki and “unaffected” *Pts*-ki/ko mice, exhibited a more severe phenotype which mimics the biochemical hallmarks of human BH_4 deficiency thus demonstrating that “affected” *Pts*-ki/ko mice are a suitable mouse model to study BH_4 deficiency.

2.3 Materials and methods

Pts Gene Targeting

A genomic clone containing the *Pts* gene was as described previously in (Turri et al., 1998), isolated from a 129/Sv-λ phage library. For targeting vector (pMSY211) construction, a 1.3 kb fragment of the *Pts* gene spanning exon 1 was used as short arm of homology (see Figure 1). A phosphoglycerate kinase promoter (*Pgk*)-Diphtheria Toxin (DT) gene cassette, essential for the negative selection of the embryonic stem (ES) cells, was added 5' to the short arm of homology. The long arm of homology was a 5.1 kb fragment containing exons 2, 3 and 4 of the *Pts* gene, and as positive selective marker, a "floxed" *Pgk*-neomycin resistance gene (neo) cassette was introduced between the short and the long arm of homology. After successful construction, the pMSY211 targeting vector was linearized and electoporated into ES cells derived from 129S6/SvEvTac strain. ES cell clones, in which homologous recombination has occurred, were confirmed by a nested PCR with 40 cycles (MSY220: 5'-GCACCCCAAGGT AGCCAAGAATTTG-3' and MSY221: 5'-TTCTTCGCCCACCCCGAAATTGATG-3') and 25 cycles (MSY226: 5'-ACCGGGCTGGAGAACATCTGATAAG-3' and MSY228: 5'-TCA GCAGCCTCTGTTCCACATACAC-3'), respectively and standard amplification conditions (not shown). For further confirmation of correctly targeted ES cell clones, Southern blot analysis was performed (not shown). One correctly targeted ES cell clone was chosen for blastocyst injection. Blastocyst injection (FVB/N host embryos) led to generation of one 50 % chimaric male that, when sexually mature, was mated with FVB females. The chimaera revealed to be germline transmitter thus resulted in the generation of heterozygous *Pts*-R15C-ki targeted mice (correct genotype was confirmed on genomic DNA from tail biopsies by *Pts*-ki/ki PCR, for details see below).

Targeting vector pMSY211 construction, blastocyst injection and generation of one chimaric male was carried out by Dr. Rossana Scavelli at the Division of Clinical Chemistry and Biochemistry (University Children's Hospital, Zürich) as well as at Institute of Laboratory Animal Science (University of Zürich). The work is described in details in PhD-dissertation "Tetrahydrobiopterin Metabolism in the Mouse: Feeding Studies and Generation of a New Mutant Model", University of Zürich, 2006.

DNA purification

Genomic mouse DNA was purified from tail biopsies by using DNeasy Blood and Tissue kit from Qiagen (Cat. no: 69506).

Genotyping of Pts-ki/ki mice from tail biopsies

Pts-R15C-ki mice were genotyped by using the following primers: HRM_F: 5'-GAGTTTGC ACTGCGCAGCCG-3' (forward) and HRM_R: 5'-AGTGCCGGTAGTGCAGCTCC-3' (reverse). The PCR-reaction, which amplified a 313 bp fragment, was performed in a final volume of 25 µl consisting of 2µl genomic DNA from tail biopsies (see *DNA purification*, 0.5 µl of each primer (10 µM), 0.5 µl 20 mM dNTPs, 0.25 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 2.5 µl 25 mM $MgCl_2$, 2.5 µl 10x A1 buffer (Solis Biodyne) and 2.5 µl S-solution (Solis Biodyne). The PCR-reaction was performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: 95°C for 15 min, 35 cycles of (95°C for 1 min, 58°C for 45 sec and 72°C for 1 min), 72°C for 10 minutes and termination at 4°C. The R15C codon exchange destroys a *BssSI* restriction site for that reason digestion with *BssSI* leads to different fragments depending on the presence of the wild-type (R15) or mutant codon (C15): the wild-type allele contains the *BssSI* restriction site therefore two fragments, whereas the mutant allele R15C has no *Bss*SI restriction site therefore one fragment. The heterozygous condition contains all three fragments.

Genotyping of *Pts*-ki/ki, *Pts*-ki/wt and wt/wt mice shown in Figure 7 is from another PCR-set-up (using MSY120: 5'-CGCTAGCTTGTGAGTCTTCG-3' and MSY121: 5'-CCTTCACCATTTCCTCTTCG-3' primers resulting in a 233 bp PCR-product before *BssSI* digestion and other reagents that are no longer commercial available) however the genotyping principle is the same with the "new" primers (HRM_F and HRM_R).

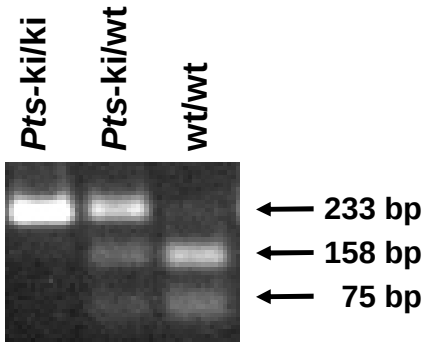

Figure 7: Genotyping of *Pts*-ki/ki, *Pts*-ki/wt and wt/wt

The wild-type allele contains a *BssSI* restriction site therefore PCR and *BssSI* digestion result in two fragments (158 bp bp and 75 bp), whereas the mutant R15C allele has no *BssSI* restriction site therefore result in one fragement (233 bp). The heterozygous condition contains all three fragments (233 bp, 158 bp and 75 bp).

The *BssSI* digested PCR fragments (PCR-product, 100xBSA, 10XNEBuffer 3 and 1000 units *BssSI* from New England Biolabs (R0587L) were incubated in 6 hours at 37°C) were run on a 2 % agarose gel and visualized under ultraviolet (UV) light in the presence of GelRed 10,000X in water (Biotium).

Genotyping of Pts-ki/ko mice from tail biopsies

Compound heterozygous (*Pts*-ki/ko) mice were genotyped by using the *Pts*-R15C-knock-in PCR described above and the *Pts*-knock-out PCR described in (Elzaouk et al., 2003). The *Pts*-knock-out PCR consists of 3 primers: MSY107: 5'-TGACTATGGGCAGAGTTGTT-3' (forward), MSY108: 5'-GATTGTTGCATTTCCCAAAC-3' (reverse) and PLACZ8: 5'-GGC -TCAGTTCGAGGTGCT-3' (reverse). The *Pts*-knock-out PCR-reaction was performed in a final volume of 25 µl containing 2 µl of genomic DNA from tail biopsies (see *DNA purification*), 0.5 µl of each primer (10 µM), 0.25 µl 20 mM dNTPs, 0.2 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 2.5 µl 25mM $MgCl_2$, 2.5 µl 10x Buffer A1 (Mg^{2+}, detergent free, Solis Biodyne) and 3 µl 10x Solution S (Solis Biodyne). The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95°C for 15 minutes, followed by 35 cycles of denaturation 95°C for 1 minute, annealing 53°C for 45 seconds and extension at 72°C for 1 minute, a final extension was performed at 72°C for 10 minutes and termination at 4°C. The PCR fragments were run on a 2 % agarose gel and visualized under ultraviolet (UV) light in the presence of GelRed 10,000X in water (Biotium).

Preparation of Mouse Tissues

Brains and livers were removed immediately from the scarified mice and snap-frozen in liquid nitrogen. Frozen harvested tissue was ground to powder and blended at 4°C with an electric homogenizer (Kinematic GmbH, Luzern, Switzerland) in cold homogenization buffer consisting of 50 mM Tris-HCl, pH 7.5/100 mM KCl/1 mM EDTA, 1 mM DTT in H_2O, 0.2 mM PMSF in isopropanol (10mg/ml), 1 µM leupeptin in H_2O (5mg/ml) and 1 µM pepstatin in methanol (1mg/ml). Tissue extracts were clarified by centrifugation at 15000 *g* for 20 min at 4°C and the supernatants were stored at minus 80°C prior to use. 4x homogenization buffer for PTPS enzyme activity assays (4 µl/mg tissue) and 10x homogenization buffer for neopterin, biopterin as well as neurotransmitter measurements (10 µl/mg tissue).

RNA extraction

RNA was extracted from mouse tissue (liver) by QIAGEN OneStep RT-PCR kit (100) from Qiagen (Cat. no: 210212).

Reverse transcription-polymerase chain reaction (RT-PCR)

The OneStep RT-PCR reaction was performed in a final volume of 50 µl (RNase-free water) containing 2 µl of RNA template, 3 µl of forward (cDNA fn: 5'-GGCGGGTGACCTTCGT-3') and reverse primer (mPTPS cDNA r n1: 5'-CGATGTTGTTGTCGGTTTCA-3') (5 µM), 2 µl Qiagen OneStep RT-PCR enzyme mix, 2 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 10 µl 5x Qiagen OneStep RT-PCR buffer and 10 µl 5x Q-solution. The RT-PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using the following conditions: 50°C for 30 minutes, 95°C for 15 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute, a final extension was performed at 72°C for 10 minutes and termination at 4°C.

The following primers (forward: R15C_F: 5'-AGCT GGGGTTTCTTTTTGGT-3' and reverse: R15C_R: 5'-GTAGCCCATGCTCCTGTCAT-3') as well as BigDye Terminator v1.1 Cycle Sequencing Kit from Applied Biosystems were used to sequence the cDNA. The sequencing PCR was performed in a 10 µl reaction mixture consisting of 0.5 µl RT-PCR product, 0.8 µl primer (5 µM), 1.5 µl BigDye and 1.25 µl 5x sequencing buffer and using standard thermal cycling: 95°C for 1 min, followed by 25 cycles (96°C for 10 sec, 50°C for 5 sec, 60°C for 3 min) and termination at 4°C. After end run 15 µl H_2O was added to the sequencing reactions before purified by gel filtration (using MultiScreen HV 96-well filter plates (Millipore) with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing results were compared with the wildtype sequence of the murine *Pts* gene (Ensembl Transcript ID: ENSMUST00000034570).

Protein Measurement

Protein concentration (g/L) was measured by using the M-TP Mikroprotein kit from Beckman Coulter Synchron LX-System (Beckman Coulter Inc, Brea, CA) (kit-no. 445860).

PTPS enzyme activity assay

The PTPS enzyme activity assay was measured in extracts from liver and brain according to (Thöny, 2008). To start, a reaction mixture consisting of 25 µl 100 mM Tris-HCl pH 7.4 (stored at room temperature), 5.5 µl 200 mM $MgCl_2$ (stored at room temperature), 5.5 µl 20 mM NADPH (freshly made), 5.5 µl 20 mM NADH (freshly made), 13.2 µl 0.5 mM NH_2P_3

(stored at -80°C), 3 µl 1 U/ml SR (stored at -80°C) and 2.3 µl 96 U/ml DHPR (stored at -20°C) was prepared. 50 µl brain lysate (liver lysate 25 µl and 25 µl 10mM Tris-HCl pH 7.4 1% Triton X-100 (stored at room temperature)) was added to the 60 µl reaction mixture and incubated for 2 hours at 37°C in darkness. The reaction was terminated by the addition of 33 µl 30 % trichloroacetic acid (TCA) (30 g TCA in 100 ml H_2O, stored at room temperature) and placed on ice for 10 minutes for protein precipitation, before centrifugation (5 min. 13000 rpm). A 20 µl 1 % iodine solution (0.1 g I2 and 0.2 g KI in 10 ml 1M HCl) was added to 100 µl of the supernatant to oxidize all the pterines to the same oxidation state. This new mixture was incubated in the dark at room temperature and after 1 hour oxidation the reaction was stopped by adding 30 µl 1 % ascorbic acid (10 mg in 1 ml H_2O, freshly made) to destroy excess iodine. After mixing, the samples were centrifuged for 10 minutes at 13000 rpm through an Amicon® Ultra-0.5 Centrifugal Filter (Millipore, Cat. no: UFC501096). The total amount of biopterin generated from the dihydroneopterin triphosphate substrate was detected by reversed-phase high-performance liquid chromatography (HPLC). Additionally, two different blanks were prepared. The first composed of 60 µl reaction mixture and 50 µl 100 mM Tris-HCl pH 7.4, the second of 60 µl 100 mM Tris-HCl pH 7.4 and 50 µl tissue lysate. Both blanks were incubated at ice for 2 hours in the dark and then treated as the rest of the samples from the 30% TCA step on. One unit of PTPS produced 1 µmol of biopterin per minute at 37°C. The activity was expressed in µU per mg protein.

Neopterin and Biopterin Measurements

Neopterin and biopterin were measured as described in (Thöny, 2008). Tissue lysates from homoganized mouse liver or mouse brain were prepared as described under "*Preparation of Mouse Tissue*". The neopterin and biopterin measurements were started by adding 10 µl 0.5 % iodine solution in 1 M HCl to 100 µl lysate (liver only 20 µl plus 80 µl homogenizations buffer followed by incubated for 1 hour at room temperature in darkness. Three reagents were added to the lysates and incubated for 1 hour at 37°C in darkness: 1) 10 µl 2 % ascorbic acid (2 mg ascorbic acid/100 µl H_2O, prepare fresh), 2) 14 µl 1 M sodium hydroxide, 3) 20 µl alkaline phosphatase solution (alkaline phosphatase was diluted 1:10 in 3.2 M ammonium sulphate solution, 1 mM $MgCl_2$, 0.1 M $ZnCl_2$, pH=7). Finally, 5 µl 2 M HCl was added to the lysates. The lysates were centrifuged for 15 minutes at 6500 rpm through Amicon® Ultra-0.5 Centrifugal Filter (Millipore, Cat. no: UFC501096) before transferred to the HPLC-system. The neopterin and biopterin concentrations were calculated as followed: (nmol/L neopterin or biopterin x 159) / (µl lysate/assay x mg/ml protein) = pmol/mg protein.

Neurotransmitter Measurements

The monoamine neurotransmitter (dopamine, HVA, serotonin and 5-HIAA) were measured as previous described in (Blau et al., 1999, Elzaouk et al., 2003). Tissue lysates from homoganized mouse brain was prepared as described under ***"Preparation of Mouse Tissue".*** 15 µl 1 M HCl was added to 150 µl brain lysate and centrifuged at 4°C for 15 minutes at 7500 rpm through Amicon® Ultra-0.5 Centrifugal Filter (Millipore, Cat. no: UFC501096) before transferred to the HPLC-system. Each monoamine neurotransmitter concentration (dopamine, HVA, serotonin and 5-HIAA) was calculated as followed: ((monoamine neurotransmitter nmol/L)/150) x (165/(mg/ml protein)) = pmol/mg protein.

Phenylalanine Concentration in the Blood

The blood from the mice was collected on Guthrie cards (filter paper card) (Guthrie and Susi, 1963). Phenylalanine (and tyrosine) concentrations (µmol/L) were measured using electrospray tandem mass spectrometry (ESI-MS/MS) (Rashed et al., 1995).

Statistical analysis

All tests were performed using GraphPad PRISM 5.0 software. Descriptive statistics including mean ± standard deviation (S.D) and Student's t-tests were used. p values <0.05 were considered significant.

Systemic phenotype analysis by the German Mouse Clinic

To obtain the required number of animals of the primary screening (80 in total consisting of 20 *Pts*-ki/ki males, 20 *Pts*-ki/ki females, 20 wt/wt males and 20 wt/wt females), we bred 45 breeding-pairs of *Pts*-ko/wt males with *Pts*-ki/wt females. All the breeding-pairs were separated after six days of mating to reduce heterogeneity of the cohort (not more than 7 days difference in age between the littermates to be phenotypical analyzed). Within the one week 237 mice were born (120 males and 117 females). In addition, 51 mice were born (25 males and 26 females) outside the mating-window consequently this cohort was kept as second choice. From the two cohorts of mice (mostly the first except for three *Pts*-ki/ki females): 20 *Pts*-ki/ki males, 20 *Pts*-ki/ki females, 20 wt/wt males and 18 wt/wt females were sent to the GMC at the age of 8 weeks for comprehensive phenotypical and behavioural characterization of the homozygous *Pts*-R15C-ki allele.

2.4 Results

2.4.1 Generation of *Pts*-ki/ki mice

In order to generate a *Pts* mouse model by 'knocking-in' a single codon exchange (*Pts*-R15C) in the *Pts* gene (*Pts*-ki) (Turri et al., 1998) a targeting vector (pMSY211) was generated (Scavelli, University of Zürich, 2006).

A

```
                                R16C
                                 ↓
Human 1MSTEGGGRRCQAQVSRRISFSASHRLYSKFLSDEENLKLFGKCNNPNGHGHNYKVVVTVH
       ::. :  :: .:..:: .:::::::::.:  ::::::::.:::::::::::::::::::::
Mouse 1MSAAGDLRR-RARLSRLVSFSASHRLHSPSLSDEENLRVFGKCNNPNGHGHNYKVVVTVH
                                 ↑
                                R15C
Human  GEIDPATGMVMNLADLKKYMEEAIMQPLDHKNLDMDVPYFADVVSTTENVAVYIWDNLQK
       :::::.::::::::.:::.::::::::.:::::::::.:::::::.:::::::::::..:::
Mouse  GEIDPVTGMVMNLTDLKEYMEEAIMKPLDHKNLDLDVPYFADAVSTTENVAVYIWESLQK

Human  VLPVGVLYKVKVYETDNNIVVYKGE 145
       .:::::.:::::::.:::::::::::
Mouse  LLPVGALYKVKVFETDNNIVVYKGE 144
```

B

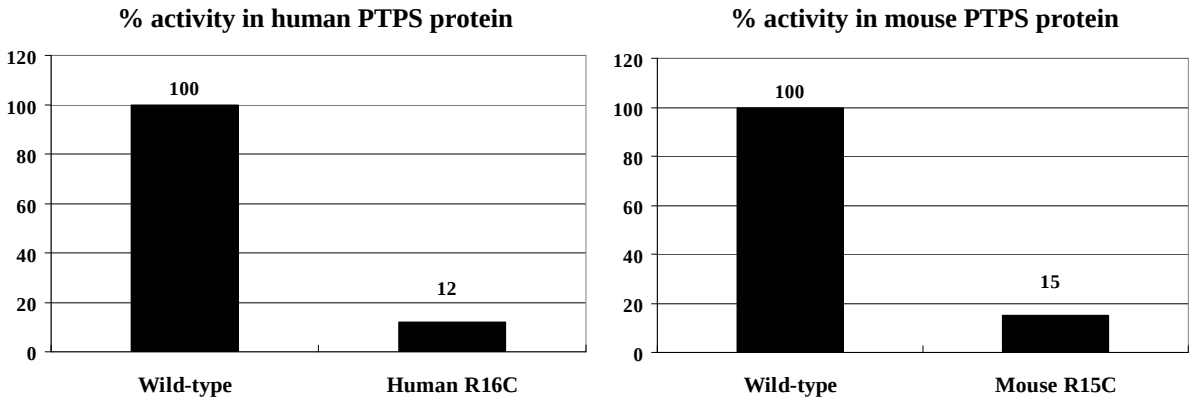

Figure 8: PTPS sequence alignment and PTPS activity of recombinant PTPS in COS-1 cells

A: Sequence alignment of human and mouse, which share 82 % sequence, identity. Mouse *Pts*-R15C is equivalent to the human *PTS*-R16C mutation. Human *PTS*-R16C and mouse *Pts*-R15C are marked in arrows. Human PTPS: NP_000308, Mouse PTPS: NP_035350. B: PTPS enzyme activity of recombinant PTPS protein in mammalian COS-1 cells revealed that mouse *Pts*-R15C has a significant reduced PTPS enzyme activity (15 %) compared to wild-type (100 %). Moreover, the mouse *Pts*-R15C (15 %) and human *PTS*-R16C (12 %) mutations demonstrated comparable PTPS enzyme activity levels. Figure B is adapted from (Oppliger et al., 1995) and (Scherer-Oppliger et al., 1999).

Arginine 15 to cysteine (*Pts*-R15C) was chosen because it is equivalent to the human *PTS*-R16C mutation which is associated with a peripheral or mild phenotype that do not synthesizes BH_4 in peripheral organs but lead to normal BH_4 and neurotransmitter levels in the CNS (Thony et al., 1994) (Figure 8). Beside, the *Pts*-R15C allele the patient was also having on the second allele a 14 bp deletion leading to a frameshift at lysine 120 and a premature stop codon (Thöny et al., 1994).

In addition, previous *in vivo* PTPS activity studies on recombinant PTPS protein transfected into mammalian COS-1 cells have shown that the mouse R15C mutation has significant reduced PTPS enzyme activity compared to wild-type but on the other hand showed comparable PTPS enzyme activity levels with the human R16C mutation (Oppliger et al., 1995, Scherer-Oppliger et al., 1999) (Figure 8).

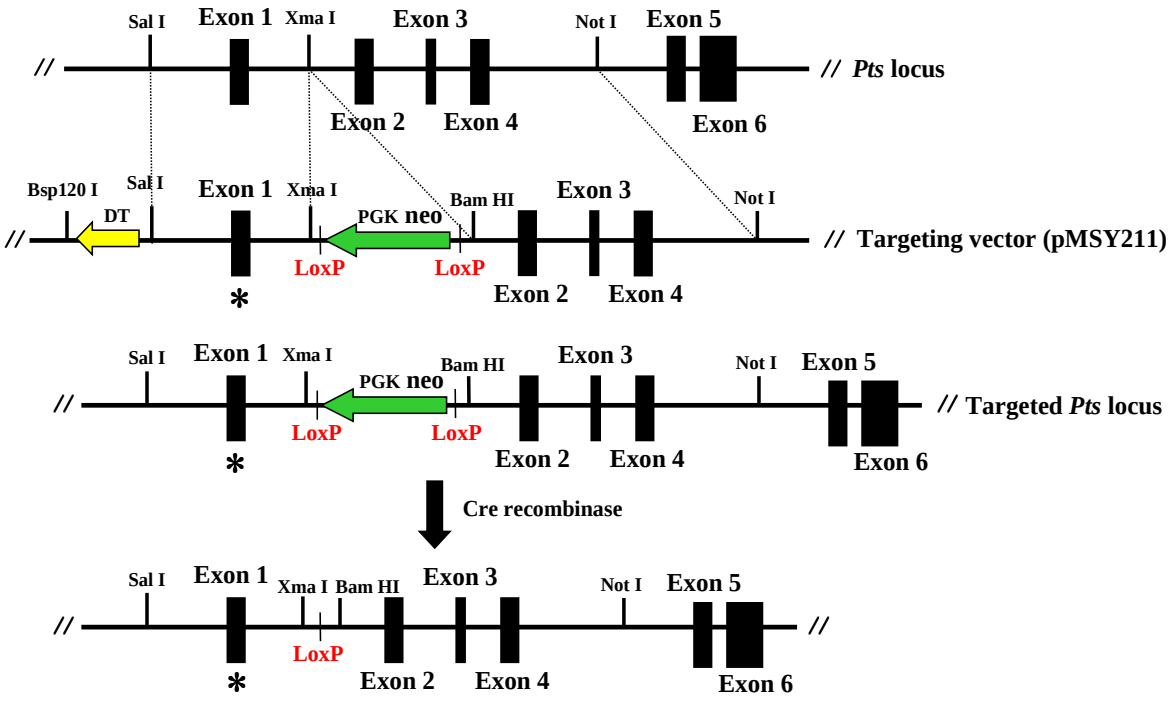

Figure 9: Targeting of *Pts* gene locus by homologous recombination in ES cells

Schematic representation of the *Pts* gene locus (top), the targeting vector including the R15C codon exchange (marked by *), a *Pgk*-DT-gene-cassette (yellow) for negative selection and a "floxed" *Pgk*-neo-gene-cassette (green) for positive selection (middle) and the targeted *Pts* locus after homologous recombination (bottom). Additionally, the "floxed" *Pgk*-neo-gene-cassette was deleted by Cre recombinase.

The targeting vector pMSY211 was constructed with a 1.3 kb fragement of the *Pts* gene, spanning exon 1 as the short arm of homology and a 5.1 kb fragment including exon 2, 3 and 4 of the *Pts* gene as the long arm of homology.
For positive and negative selection, a *Pgk*-DT-gene-cassette was added 5' to the short arm of homology and a "floxed" *Pgk*-neo-cassette was introduced between the short and long arm of homology, respectively. The targeting strategy for introducing the pMSY211 targeting vector into ES cells by homologous recombination is depicted in Figure 9.
Correct homologous recombination event in neomycin-resistant ES cells was identified by nested PCR (not shown) as well as Southern blot analysis using a 5' probe (upstream to the targeting vector) and a 3' probe (inside the targeting vector) (not shown) (Scavelli, University of Zürich, 2006). One correctly targeted ES cell clone was injected by blastocyst injection into FVB/N host embryos, which led to the generation of one chimera male (50 % chimeric) and subsequently to heterozygous *Pts*-R15C-ki targeted mice (*Pts*-ki/wt) due to germline transmission. *Pts*-ki/wt mice revealed to be viable, fertile and phenotypical normal thus the heterozygous *Pts*-R15C-ki allele was breed to homozygosity (*Pts*-ki/ki) (Scavelli, University of Zürich, 2006).

Before the newly generated *Pts*-ki/ki and *Pts*-ki/wt mice were subjected to a broad range of biochemical analysis for characterization, the expression of the R15C codon in the transgenic mouse tissue (liver and brain) was confirmed by RT-PCR and cDNA sequencing (not shown).

Due to the pronounced observations within homozygous *Pts*-ko mice among this newborn lethality (Elzaouk et al., 2003, Sumi-Ichinose et al., 2001) (see also Introduction: Mouse models in the BH_4 metabolism), the overall condition of the *Pts*-ki/ki mice in terms of weight, movement, behaviour, sexual maturation and fur was carefully observed. Our breeding experiments revealed that *Pts*-ki/ki mice in contrast to homozygous *Pts*-ko mice are viable, fertile and showed no noticeable abnormalities concerning weight, movements, behaviour or fur compared to *Pts*-ki/wt and wt/wt mice from the same litter.

2.4.2 Biochemical characterization of *Pts*-ki/ki

The biochemical hallmarks of human BH_4 deficiency due to mutations in the *PTS* gene, are characterized by very low or absent of PTPS enzyme activity, high neopterin levels, very low or not detectable biopterin levels, HPA (elevated blood phenylalanine) as well as monoamine neurotransmitter deficiency in the CNS (exclusive for the severe/central phenotype of PTPS deficiency) (Campeau et al., 2007).

Therefore, we investigated PTPS enzyme activity (liver and brain), neopterin and biopterin levels (liver and brain), blood phenylalanine concentration as well as brain monoamine neurotransmitters levels within *Pts*-ki/ki, *Pts*-ki/wt and wt/wt mice to see whether the *Pts*-ki/ki phenotype would mimic the biochemical hallmarks for human BH_4 deficiency.

As indicated in Figure 10A, the *Pts*-ki/ki mice had a significant reduced PTPS enzyme activity both in liver and brain compared to *Pts*-ki/wt and wt/wt mice.

Neopterin measurements by HPLC analysis revealed highly elevated neopterin levels in the *Pts*-ki/ki mice compared to the *Pts*-ki/wt mice (Figure 10B). Whereas, the neopterin levels were detectable within liver and brain tissue from *Pts*-ki/ki and *Pts*-ki/wt mice, the neopterin levels within liver and brain tissue from wt/wt mice were not detectable (Figure 10B). In contrast to the neopterin, the brain and liver biopterin levels showed, no variations among the three measured genotypes (*Pts*-ki/ki, *Pts*-ki/wt and wt/wt) (Figure 10C).

Reduced biopterin is a common feature for almost all human BH_4 deficiencies except SR and DHPR deficiency (Longo, 2009) thus the *Pts*-ki/ki biochemical phenotype clearly diverges from the phenotypes associated with human BH_4 deficiency.

Blood phenylalanine concentration were measured from Guthrie cards using electrospray tandem mass spectrometry (ESI-MS/MS) (Rashed et al., 1995). The ESI-MS/MS results revealed no significant differences in the blood phenylalanine concentrations between the *Pts*-ki/ki, *Pts*-ki/wt and wt/wt mice (Figure 10D). Moreover, none of the three groups showed HPA (Elzaouk et al., 2003).

This encouraged us to challenge the mice with two different concentrations (30 mg/L and 30 g/L) of phenylalanine in the drinking water for 5 days in order to investigate whether a phenylalanine challenge would lead to HPA or whether the mice would be able to cope with the increased intake of phenylalanine in the drinking water (on standard mouse chow (KLIBA NAFAG diet no. 3430) the daily intake of pheylalanine is approximately 25-50 mg/day). The exact amount of phenylalanine received via the drinking water (average 4-10 ml water/day) was not controlled.

As it appears in Figure 10D, the *Pts*-ki/ki mice showed no indications of HPA when challenged with two different concentration of phenylalanine in the drinking water.

In addition, we analyzed the monoamine neurotransmitters serotonin and dopamine plus their end-metabolites 5-HIAA and HVA, respectively in brain tissue to investigate whether the *Pts*-ki/ki mice were subjected to monoamine neurotransmitter deficiency in the CNS.

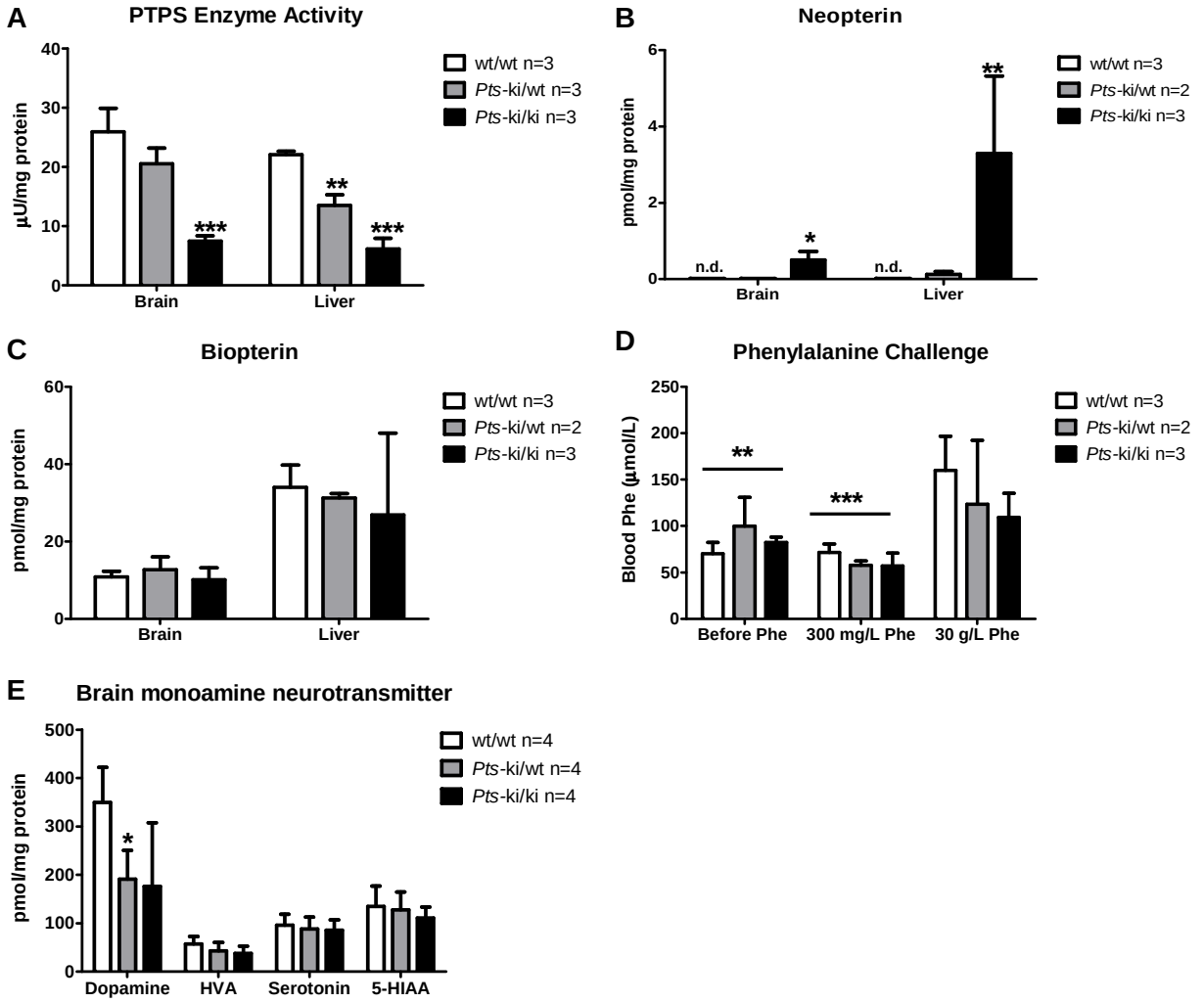

Figure 10: Biochemical characterization of liver and brain tissue from *Pts*-ki/ki, *Pts*-ki/wt and wt/wt mice

A: PTPS enzyme activity (PTPS/protein uU/mg) in liver and brain. B: Neopterin levels (pmol/mg protein) in liver and brain. C: Biopterin levels (pmol/mg protein) in liver and brain. D: Blood phenylalanine concentrations (µmol/L) before and after phenylalanine challenge with 300 mg/L Phe and 30 g/L Phe, respectively in the drinking water for 5 days. E. Brain monoamine neurotransmitters (dopamine and serotonin) and their end-metabolites (HVA and 5-HIAA) (pmol/mg protein). The genotypes are indicated by the bar colour: wt/wt (white), *Pts*-ki/wt (grau) and *Pts*-ki/ki (black). n.d. indicates not detectable. Values are the mean ± S.D from n independent measurements. Significant difference from the corresponding value for wild-type is indicated by asterisks: *, $p < 0.05$, **, $p < 0.01$, ***, $p < 0.001$, ****, $p < 0.0001$ (Student's *t* test).

As seen in Figure 10E, no significant differences were shown neither in the monoamine neurotransmitter levels nor their end-metabolites between *Pts*-ki/ki, *Pts*-ki/wt and wt/wt mice (*Pts*-ki/wt showed significant reduced dopamine compared to *Pts*-ki/ki and wt/wt). These findings indicated that *Pts*-ki/ki mice, unlike homozygous *Pts*-ko mice did not suffering from brain monoamine neurotransmitter deficiency (Figure 10E).

2.4.3 Systemic phenotype analysis by the German Mouse Clinic

For further comprehensive phenotypical and behavioural characterization of *Pts*-ki/ki mice, we decided to collaborate with the German Mouse Clinic (GMC) in Munich, Germany (Fuchs et al., 2009, Beckers et al., 2009, Fuchs et al., 2011, Gailus-Durner et al., 2005). Over a period of 10 weeks 78 mice (20 *Pts*-ki/ki males, 20 *Pts*-ki/ki females, 20 wt/wt males and 18 wt/wt females) were subdivided into two pipelines and subjected to numerous non-invasive tests including measurements of approximately 550 parameters within 14 different areas (Figure 3). The results of the primary screening are outlined in the next paragraphs and in Supplementary Information.

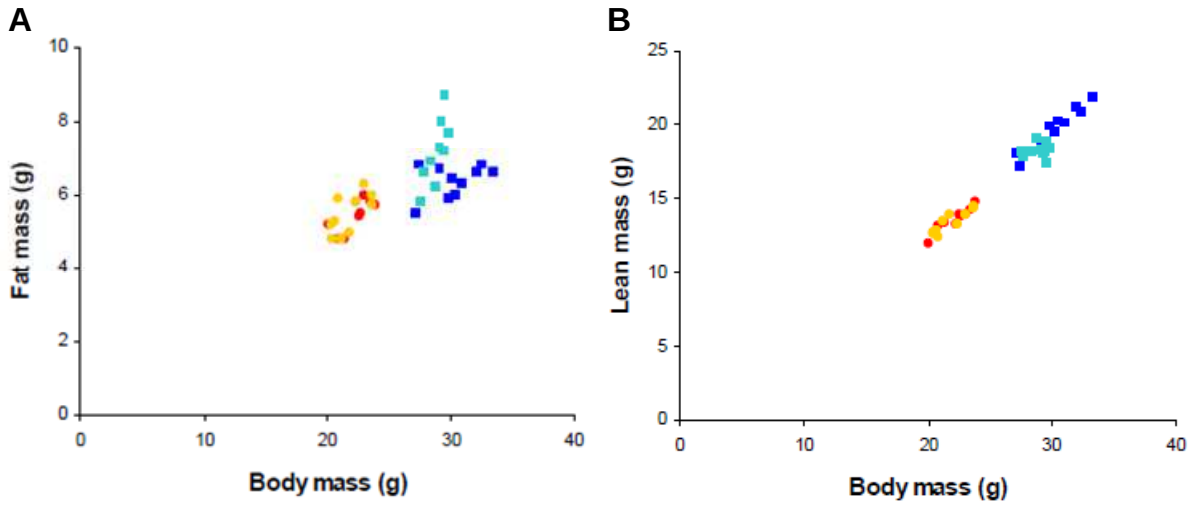

Figure 11: Body composition analysis by non-invasive NMR

Non-invasive NMR scans were used to determine the fat mass (g), lean mass (g) and body mass (g) in the *Pts*-ki/ki mutant mice (both males and females) compared to wt/wt control mice. The *Pts*-ki/ki mice used for NMR scans had a significant reduced body mass (both males and females) and sex-specific shift in body composition as the *Pts*-ki/ki mutant males had a higher body fat mass and lower lean mass. Wt/wt control female (red), *Pts*-ki/ki mutant females (orange), wt/wt control males (dark-blue) and *Pts*-ki/ki mutant males (light-blue). Data, table and figures provided by Dr. Jan Rozman from the GMC.

The body composition analysis by non-invasive NMR revealed that *Pts*-ki/ki mice have a significantly reduced body mass compared to the wt/wt control mice. However, in *Pts*-ki/ki males the body fat content was found to be significantly increased and the lean mass to be significantly decreased (Figure 11). No shift in body composition was detected in *Pts*-ki/ki females (Figure 11).

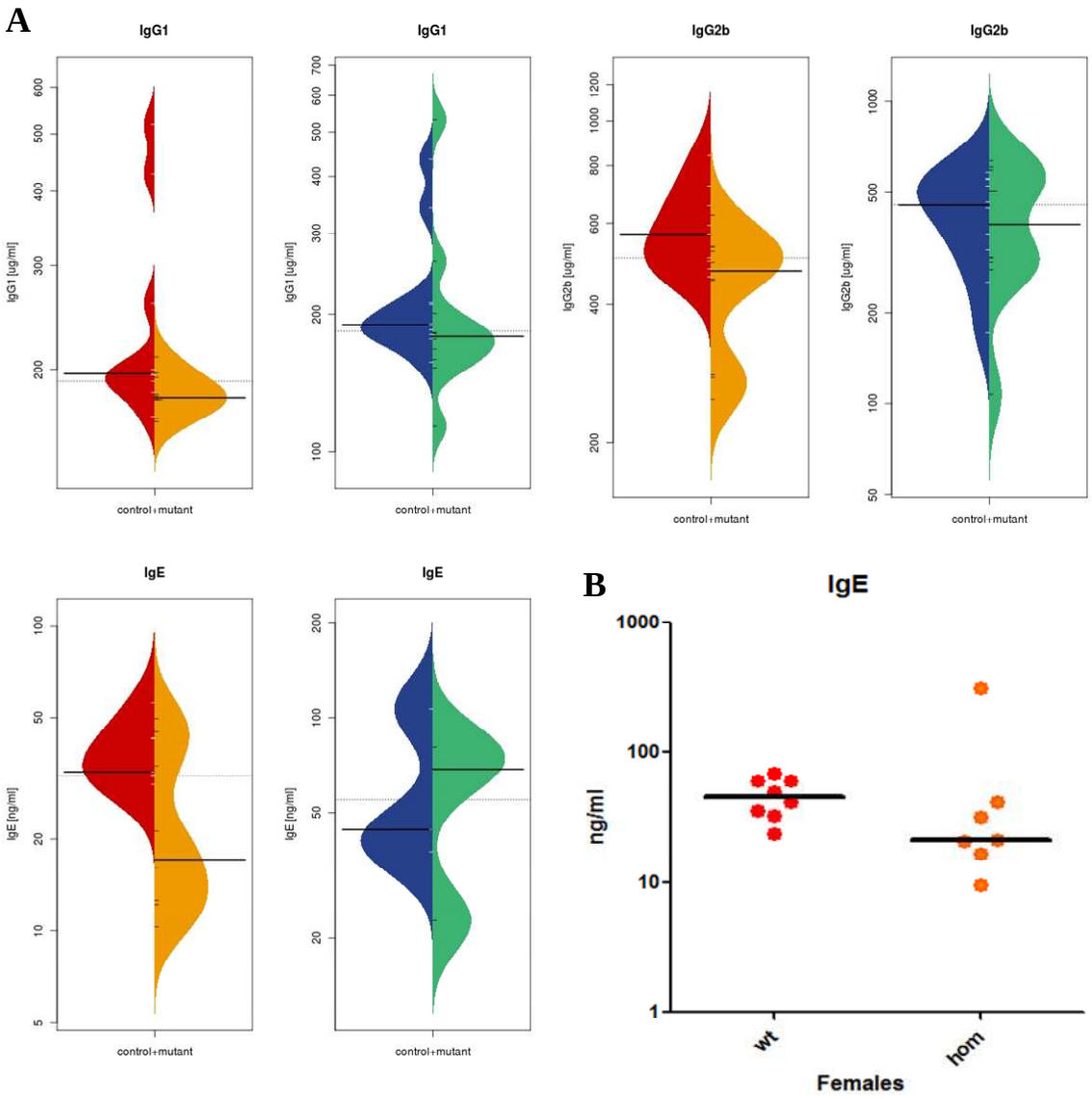

Figure 12: IgG1, IgG2b and IgE immunoglobulins in *Pts*-ki/ki mice compared to wt/wt

A: The immunology and allergy screen showed slightly reduced levels of immunoglobulins IgG1, IgG2b and IgE in *Pts*-ki/ki mutant females (orange) compared to wt/wt control females (red). This observation was not found in either wt/wt control males (blue) or *Pts*-ki/ki mutant males (green). B: The plasma IgE levels of the female mice (wt/wt control females (red/wt) and *Pts*-ki/ki mutant females (orange/hom)) were repeated revealing the same tendency as first time. Due to the slightly reduced levels of IgG1, IgG2b and IgE in *Pts*-ki/ki females a secondary test with a new cohort of mice (n=10 to 12 per sex per group) to confirm this observation has been suggested. Data, table and figures provided by Dr. Antonio Aguilar Pimentel at the GMC.

The immunology and allergy screen showed no changes in the frequencies of most leukocyte subsets in peripheral blood but only subtle differences in the frequency of $CD11b^+$ expressing NK cells (a maturation marker for NK cells) and slightly lower levels of immunoglobulins IgG1, IgG2b and IgE in *Pts*-ki/ki female mice (Figure 12A). The immunoglobulin IgE concentration were re-measured in plasma samples from female mice (wt/wt and *Pts*-ki/ki) and also the second time the same tendency was observed (Figure 12B). Due to the lower levels of IgG1, IgG2b and IgE in *Pts*-ki/ki females a secondary test with a new cohort of mice (n=10 to 12 per sex per group) to confirm the observed phenotype was suggested.

Taken together, the primary screening of the *Pts*-ki/ki mice at the GMC led to many interesting observations (see also Supplementary Information). Two of these were more pronounced namely the indication that *Pts*-ki/ki males have an increased body fat mass and decreased lean mass compared to wt/wt males (Figure 11) and that *Pts*-ki/ki females have a slightly reduced IgG1, IgG2 and IgE levels than wt/wt females (Figure 12). For confirmation of these findings, secondary screenings with a new cohort of mice within energy metabolism and immunology have been suggested.

2.4.4 Abdominal obesity in *Pts*-ki/ki mice on standard mouse chow and high fat diet

A previous observation in our laboratory have shown that *Pts*-ko/wt males (n=4) upon feeding with high fat diet (58 kcal% fat with sucrose compared to normal diet with 11 kcal% fat with corn starch) over a period of several weeks increases their intra-abdominal adipose tissue (sum of epididymal and perigonadal fat) compartments two-fold compared to 1.6-fold increase in wt/wt males (Table 1). Yet, in a standard oral glucose tolerance loading test there was no difference in glucose clearance between *Pts*-ko/wt and wt/wt mice.

Table 1: Intra-abdominal fat tissue in *Pts*-ko/wt and wt/wt mice fed with high fat diet

Males	**Diet**	**Body weight in g (increase)**		**Intra-abdominal fat in mg (increase)**	
Pts-ko/wt (n = 4)	normal	3.63 ± 1.24	(100 %)	421.25 ± 121.21	(100 %)
Pts-ko/wt (n = 4)	high fat	9.98 ± 1.20	(275 %)	845.50 ± 169.73	(201 %)
wt/wt (n = 4)	normal	4.70 ± 0.91	(100 %)	342.50 ± 142.71	(100 %)
wt/wt (n = 4)	high fat	11.20 ± 2.16	(238 %)	542.25 ± 170.12	(158 %)

To confirm, this observation on a larger amount of *Pts*-ko/wt mice and to include *Pts*-ki/ki mice, we breed 20 wt/wt males, 20 *Pts*-ko/wt males as well as 20 *Pts*-ki/ki males and subjected them in collaboration with Professor Christian Wolfrum at Institute of Food Nutrition and Health at ETH Zürich to standard mouse chow or high fat diet (Research Diets, Inc. D12331: 58 kcal% fat w/sucrose Surwit Diet) for 10 weeks (ad libitum).

The average increase/gain in body weight (gram) was measured at two time points: after 4 weeks and after 10 weeks. In addition, blood glucose levels (mM) were measured after 4 weeks and 10 weeks, respectively in the mice subjected to high fat diet.

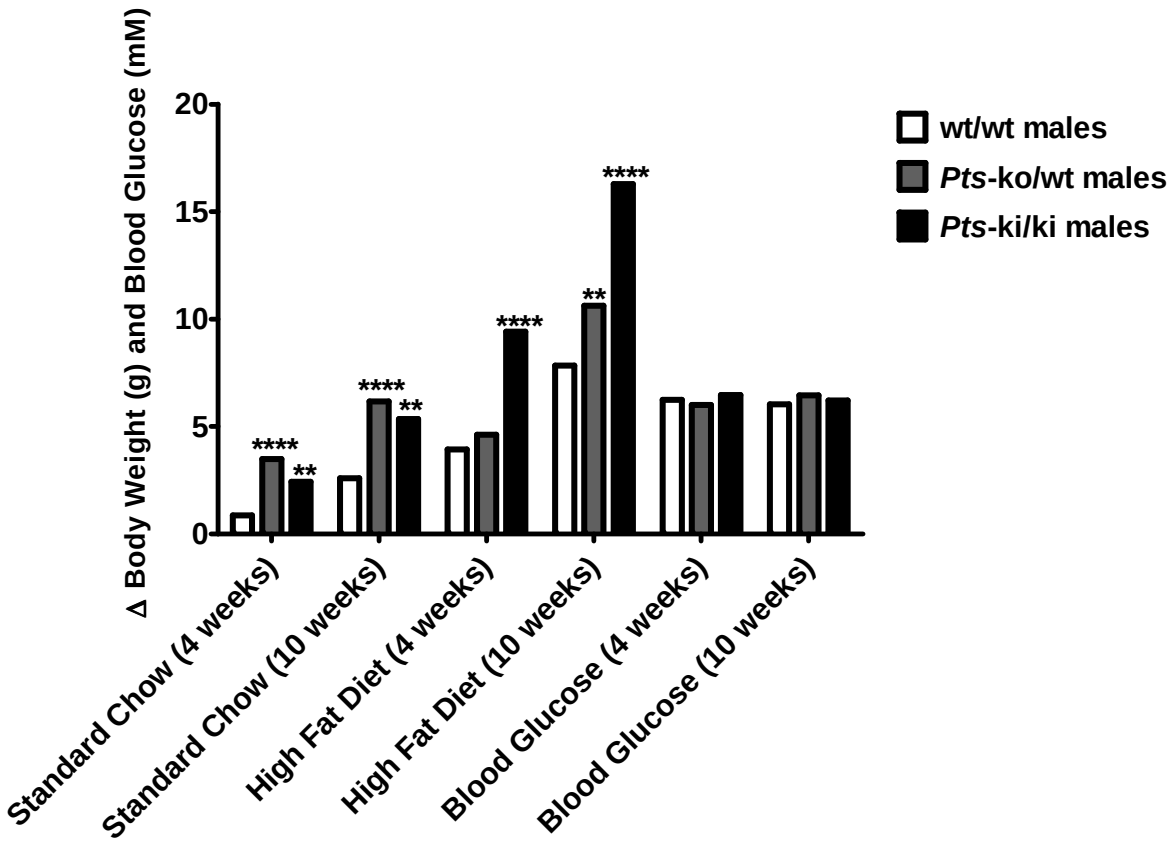

Figure 13: Average gain in body weight after 10 weeks of standard chow or high fat diet

To confirm whether *Pts*-ko/wt and *Pts*-ki/ki mice have an abnormal body fat distribution, 60 mice (20 wt/wt, 20 *Pts*-ko/wt and 20 *Pts*-ki/ki) were subjected to either standard mouse chow (10 of each genotype) or high fat diet (10 of each genotype), respectively for 10 weeks. The average △/gain in body weight (gram) was measured at two different time points: after 4 weeks and after 10 weeks. In addition, blood glucose levels (mM) were measured after 4 weeks and after 10 weeks, respectively in the mice subjected to high fat diet. The genotypes are indicated by the bar colour wt/wt (white column), *Pts*-ko/wt (dark-gray column) and *Pts*-ki/ki (black column). Values are the mean ± S.D. Significant difference from the corresponding value for wild-type is indicated by asterisks: **, $p < 0.01$, ***, $p < 0.001$ (Student's *t* test).

As shown in Figure 13, we found that *Pts*-ki/ki male mice had a significantly increase in body weight at both type of diets (standard mouse chow and high fat diet) and at both time points (after 4 weeks and 10 weeks). Similar to the *Pts*-ki/ki mice, the *Pts*-ko/wt had a significant increase in body weight when subjected to high fat diet after both 4 weeks and 10 weeks.

In contrast to the *Pts*-ki/ki mice, the *Pts*-ko/wt male mice only gained significant body weight on standard mouse chow after 10 weeks but not after 4 weeks.

The blood glucose levels measured in the mice subjected to the high fat diet remained unchanged both after 4 weeks and after 10 weeks indicating that the blood glucose is not responsible for the abnormal fat distribution observed in the *Pts*-ko/wt and *Pts*-ki/ki mice.

2.4.5 Generation and characterization of compound heterozygous *Pts*-ki/ko mice

Compound heterozygous *Pts*-ki/ko mice were generated by breeding the *Pts*-R15C-ki-allele into the *Pts*-ko-allele to investigate whether *Pts*-ki/ko mice would mimic the severe/central phenotype of human BH_4 deficiency but without newborn lethality.

Due to the severe conditions observed within the homozygous *Pts*-ko mice (newborn lethality) (Elzaouk et al., 2003), the newborn *Pts*-ki/ko mice were carefully observed the first hours and days after birth for two reasons: firstly, to see whether the *Pts*-ki/ko allele would also give rise to newborn lethality and secondly, to observe the overall condition of the *Pts*-ki/ko mice in terms of body size, milk-supply (white spot in the belly), movement and behaviour.

The first hours after birth no difference between the newborn mice was observed as they showed similar body size, milk-supply, movement and behaviour (Figure 14). Yet, at day 1-2 enormous diversities were observed among the mice.

The majority of the mice showed no apparent phenotypical characteristics however a few mice stood out from their littermates as they were smaller in body size and showed isolated behaviour (alone and away from the nest) as well as inactive/passive movements (Figure 14A). The following days, several of the “affected” mice were found either dead or eaten up and for that reason we sacrificed the “affected” mice which were not already dead due to their severe condition. Genotyping of the “affected” mice (dead, eaten up and sacrificed at day 3-4) revealed that they were all *Pts*-ki/ko mice.

At 3-4 weeks of age the “unaffected” mice (no apparent phenotype) were sacrificed and shown by genotyping to be wt/wt, *Pts*-ko/wt, *Pts*-ki/wt as well as *Pts*-ki/ko mice.

Our results suggested that the *Pts*-ki/ko mice give rise to two phenotypes meaning that some of them die 3-4 days after birth (called "affected" *Pts*-ki/ko) whereas others survived to at least 3-4 weeks of age (called "unaffected" *Pts*-ki/ko).

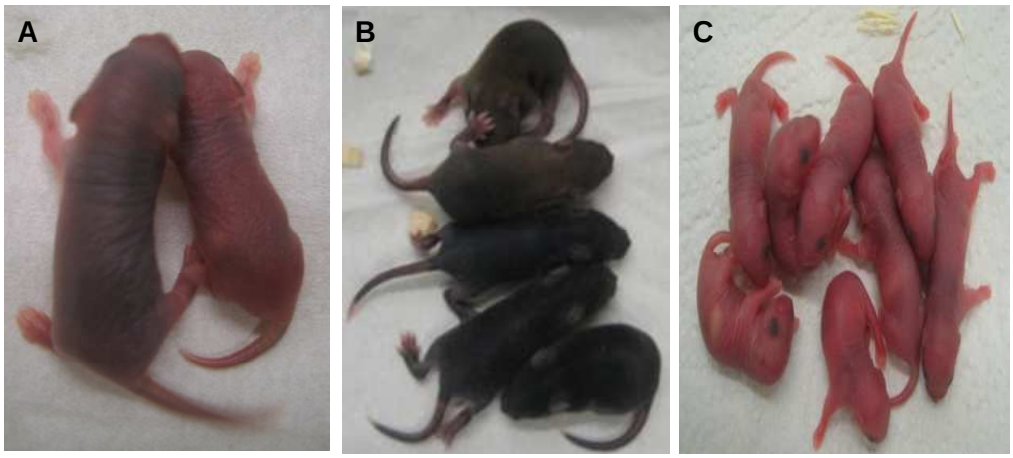

Figure 14: "Affected" *Pts*-ki/ko, "unaffected" *Pts*-ki/ko and newborn mice

A: 3-4 days after birth"affected" *Pts*-ki/ko (right) mice stood out from their "unaffected" *Pts*-ki/ko (left) littermates. B: "unaffected" *Pts*-ki/ko mice showed no apparent phenotype and survived until sacrificed at 3-4 weeks of age. C: Newborn mice were collected at day 0 as no apparent phenotypical differences appeared between the *Pts*-ki/ko mice (sum of "affected" and "unaffected" *Pts*-ki/ko) at this time point.

Subsequently, tissue (brain and liver) as well as blood collected from the sacrificed mice were subjected to biochemical analysis in order to characterize the "affected" and "unaffected" *Pts*-ki/ko mice, respectively and to investigate whether the "affected" and/or the "unaffected" *Pts*-ki/ko mice mimic human BH_4 deficiency.

As shown in Figure 15A, the liver and brain PTPS enzyme activity were significant reduced in both the "affected" and "unaffected" *Pts*-ki/ko mice compared to their *Pts*-ko/wt, *Pts*-ki/wt and wt/wt littermates. Moreover, the neopterin and biopterin concentrations were found to be considerable different in the "affected" *Pts*-ki/ko mice compared to the rest of the measured genotypes ("unaffected" *Pts*-ki/ko, wt/wt, *Pts*-ki/wt and *Pts*-ko/wt) as the "affected" *Pts*-ki/ko mice showed elevated neopterin and significant reduced biopterin in both liver and brain (Figure 15C). The phenylalanine measurements demonstrated a mild HPA (~900 µmol/L) in the "affected" *Pts*-ki/ko mice (Figure 15D). In contrast, the "unaffected" *Pts*-ki/ko mice showed normal blood phe levels (Figure 15D).

Furthermore, the monoamine neurotransmitter measurements in brain tissue from "affected" *Pts*-ki/ko mice demonstrated that they in contrast to "unaffected" *Pts*-ki/ko mice exhibit monoamine neurotransmitter deficiency (Figure 15E).

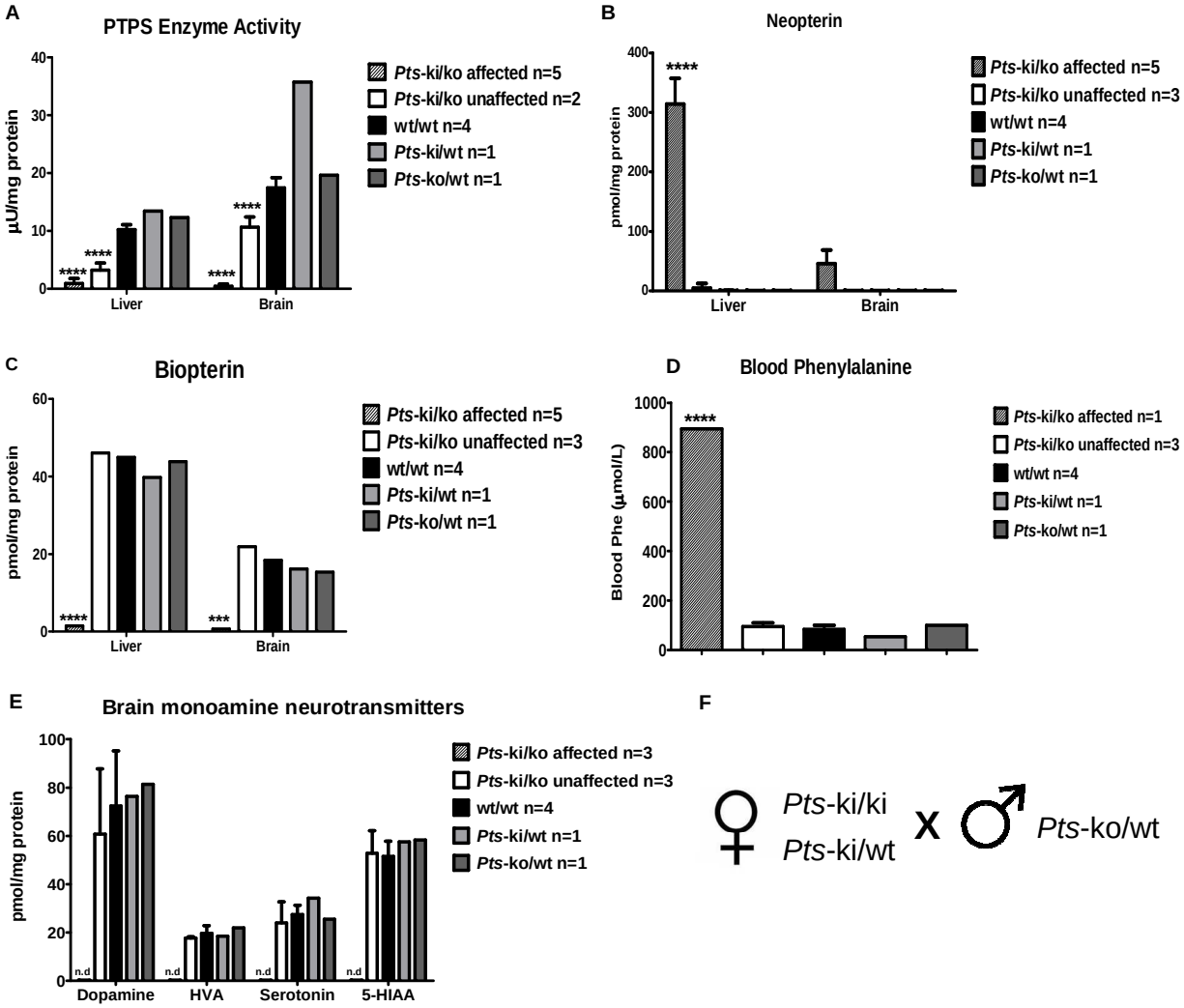

Figure 15: Biochemical characterization of compound heterozygous *Pts*-ki/ko mice

A: PTPS enzyme activity (PTPS/protein uU/mg) in liver and brain. B: Neopterin levels (pmol/mg protein) in liver and brain. C: Biopterin levels (pmol/mg protein) in liver and brain. D: Blood phenylalanine (µmol/L). E: Brain monoamine neurotransmitters (dopamine and serotonin) and their end-metabolites (HVA and 5-HIAA) (pmol/mg protein). F: Compound heterozygous *Pts*-ki/ko mice from *Pts*-ki/ki or *Pts*-ki/wt females crossed with *Pts*-ko/wt males. The genotypes are indicated by the bar colour: 3-4 days old "Affected" *Pts*-ki/ko (striped column), 3-4 weeks old "unaffected" *Pts*-ki/ko (white column), wt/wt (black column), *Pts*-ki/wt (light-gray column) and *Pts*-ko/wt (black column). Values are the mean ± S.D. n.d. indicates not detectable. Significant difference from the corresponding value for wild-type is indicated by asterisks: *** $p < 0.001$, **** $p < 0.0001$ (Student's *t* test).

2.4.6 Characterization of newborn *Pts*-ki/ko mice

Newborn *Pts*-ki/ko, *Pts*-ki/wt, *Pts*-ko/wt and wt/wt mice were scarified at day 1 to biochemical characterize them before the subdivision into the two distinguished phenotypes (sum of "affected" and "unaffected" *Pts*-ki/ko). At this time point no apparent phenotypical differences appeared between the mice in terms of size, white-belly, movement or behaviour (Figure 14C).

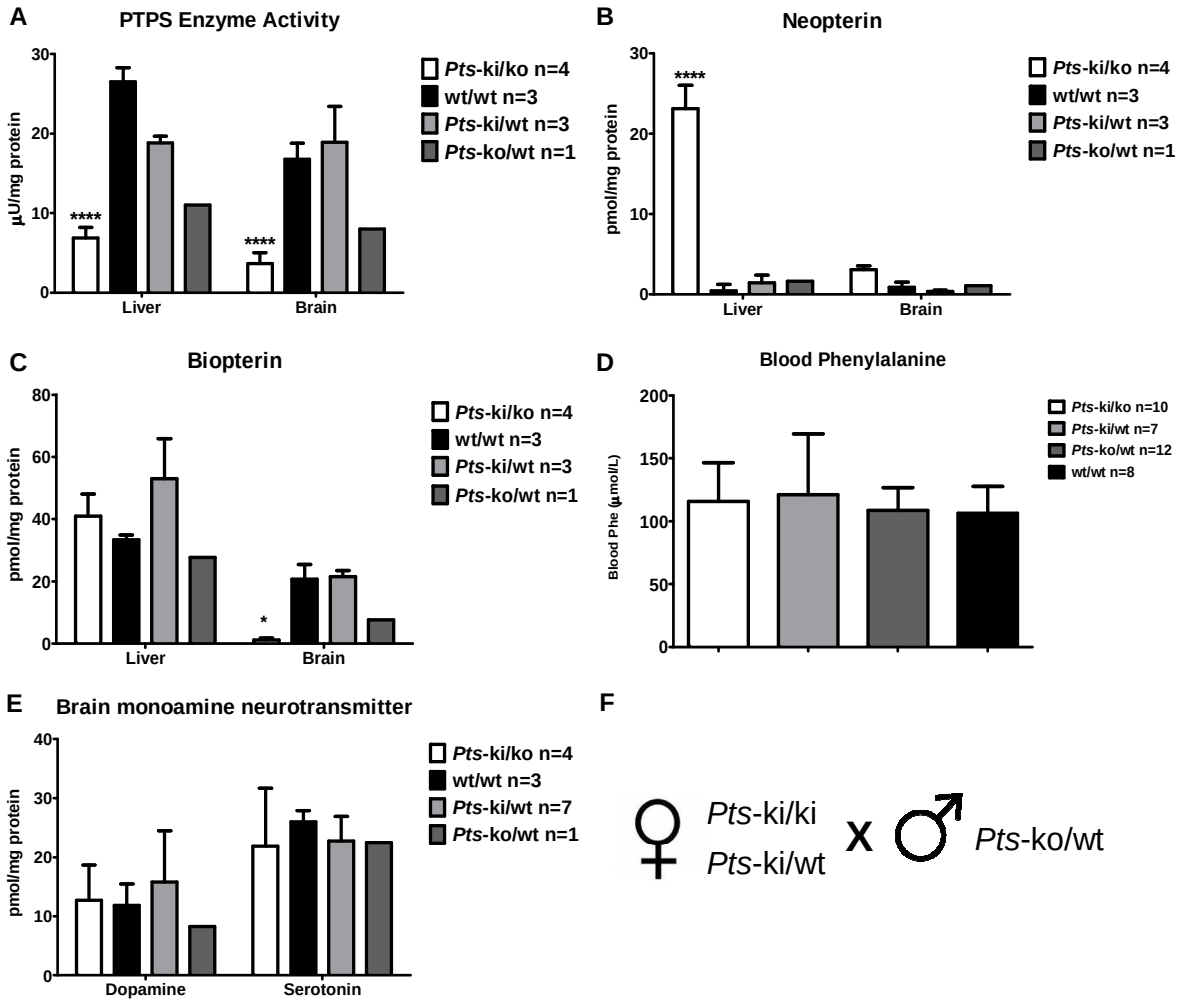

Figure 16: Biochemical characterization of newborn *Pts*-ki/ko mice

A: PTPS enzyme activity (PTPS/protein uU/mg) in liver and brain. B: Neopterin (pmol/mg protein) in liver and brain. C: Biopterin (pmol/mg protein) in liver and brain. D: Blood phenylalanine (µmol/L). E: Brain monoamine neurotransmitters (dopamine and serotonin) (pmol/mg protein). F: Newborns *Pts*-ki/ko mice from *Pts*-ki/ki or *Pts*-ki/wt females crossed with *Pts*-ko/wt males. The genotypes are indicated by the bar colour *Pts*-ki/ko (white column), wt/wt (black column), *Pts*-ki/wt (light-gray column) and *Pts*-ko/wt (dark-gray column). Values are the mean ± S.D. Significant difference from the corresponding value for wild-type is indicated by asterisks: * $p < 0.05$, *** $p < 0.001$ (Student's *t* test).

As shown in Figure 16A, the PTPS enzyme activity was significantly reduced in liver and brain within the newborn *Pts*-ki/ko mice in contrast to its wt/wt, *Pts*-ki/wt and *Pts*-ko/wt littermates. Whereas, the liver neopterin levels were found to be significantly increased in the newborn *Pts*-ki/ko mice (Figure 16B), biopterin levels revealed no significant differences compared to the three other genotypes (newborn wt/wt, *Pts*-ki/wt or *Pts*-ko/wt) (Figure 16C). Unlike the brain neopteirn levels (Figure 16B), the brain biopterin levels within the newborn *Pts*-ki/ko mice were significant reduced compared to newborn wt/wt, *Pts*-ki/wt or *Pts*-ko/wt mice (Figure 16C).

The blood phenylalanine concentrations measured from Guthrie cards by ESI-MS/MS revealed no sign of HPA in the newborn *Pts*-ki/ko mice (Figure 16D). Likewise, no pronounced differences were observed in the blood phenylalanine concentrations among the *Pts*-ki/wt, *Pts*-ko/wt and wt/wt mice (Figure 16D).

To test whether newborn *Pts*-ki/ko mice were suffering from monoamine neurotransmitter deficiency in the CNS, we analyzed the brain tissue for dopamine and serotonin. No variations were shown in the brain monoamine neurotransmitters between the four genotypes (Figure 16E), indicating that newborn *Pts*-ki/ko mice are not suffering from monoamine neurotransmitter deficiency in the CNS.

2.4.7 Breeding of *Pts*-ki/ki or *Pts*-ki/wt males with *Pts*-ko/wt females

To reproduce, elaborate and investigate the “affected” compound heterozygous phenotype in more details, we breed *Pts*-ki/ki or *Pts*-ki/wt males with *Pts*-ko/wt females.

During the first days after birth, we noticed that the “affected” *Pts*-ki/ko phenotype, which was generated when *Pts*-ki/ki or *Pts*-ki/wt females were crossed with *Pts*-ko/wt males, did not arise when *Pts*-ki/ki or *Pts*-ki/wt males were mated with *Pts*-ko/wt females.

All the mice born (10 *Pts*-ki/ko, 7 *Pts*-ki/wt, 12 *Pts*-ko/wt as well as 8 wt/wt) survived and showed no indication of phenotypical abnormalities (normal body size, milk-supply (white-belly), behaviour and movements) until sacrificed at the age of three weeks.

The biochemical analyses of the four obtained genotypes, including measurements of blood phenylalanine, neopterin (liver and brain), biopterin (liver and brain) as well as brain monoamine neurotransmitters demonstrated no differences in the blood phenylalanine levels among the mice likewise no indications on HPA (Figure 17). With reference to the neopterin levels measured in the liver and brain, the *Pts*-ki/ko mice revealed a significant elevated neopterin in the liver compared to the *Pts*-ki/wt, *Pts*-ko/wt and wt/wt mice (Figure 17B).

In comparison, no diversities were observed between the mice within the brain neopterin just as the biopterin levels both in liver and brain were the same in the four genotypes (*Pts*-ki/ko, *Pts*-ki/wt, *Pts*-ko/wt and wt/wt) (Figure 17C). Moreover, we found no significant variety in the brain monoamine neurotransmitter levels within the *Pts*-ki/ko mice compared to the *Pts*-ki/wt, *Pts*-ko/wt and wt/wt mice thereby demonstrating that *Pts*-ki/ko mice born from *Pts*-ki/ki or *Pts*-ki/wt males and *Pts*-ko/wt females do not exhibit monoamine neurotransmitter deficiency in the CNS (Figure 17D).

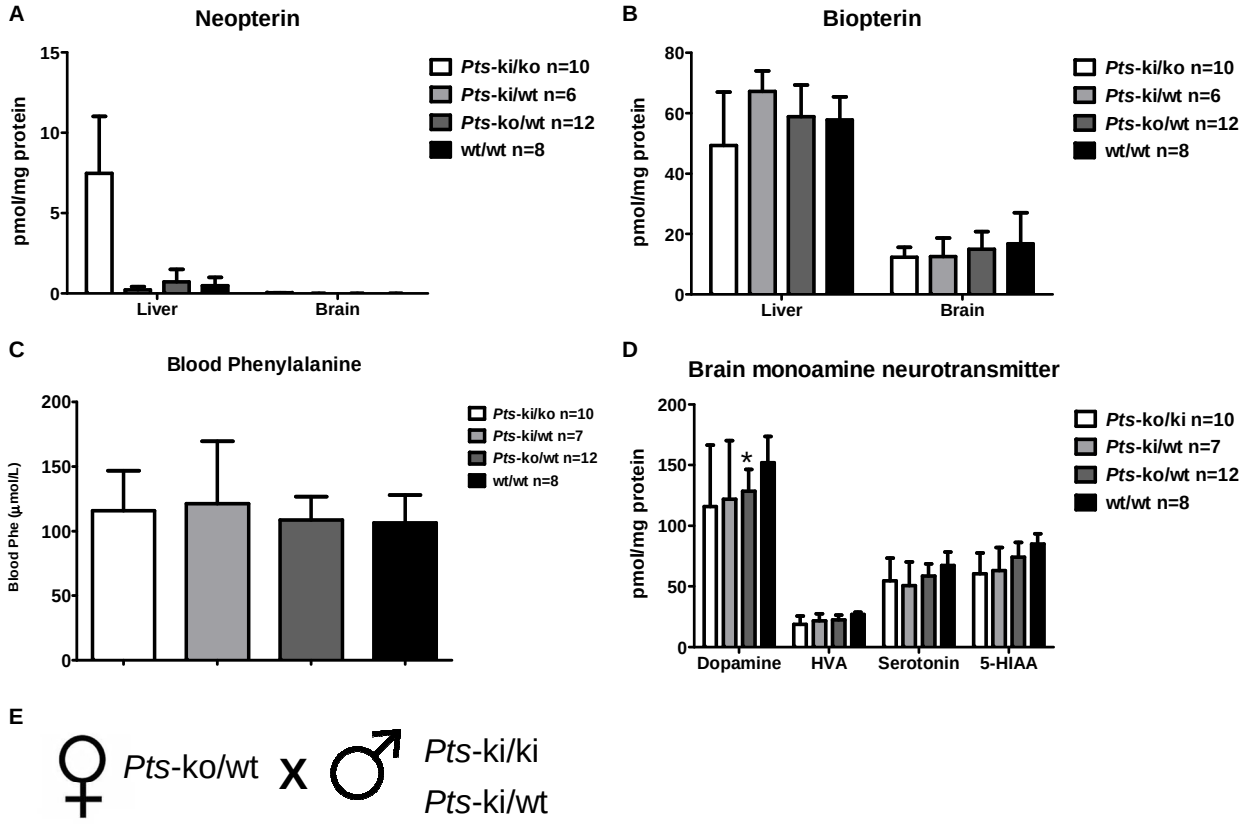

Figure 17: Biochemical characterization of 3 weeks old *Pts*-ki/ko mice after crossing *Pts*-ki/ki or *Pts*-ki/wt males with *Pts*-ko/wt females

A: Neopterin levels (pmol/mg protein) in liver and brain. B: Biopterin levels (pmol/mg protein) in liver and brain. C: Blood phenylalanine (µmol/L). D: Brain monoamine neurotransmitters (dopamine and serotonin) and their end-metabolites (HVA and 5-HIAA) (pmol/mg protein). E: *Pts*-ki/ko mice from *Pts*-ko/wt females and *Pts*-ki/ki or *Pts*-ki/wt males. The genotypes are indicated by the bar colour: *Pts*-ki/ko (white column), wt/wt (black column), *Pts*-ki/wt (light-gray column) and *Pts*-ko/wt (dark-gray column). Values are the mean ± S.D. ND indicates Not detectable. Significant difference from the corresponding value for wild-type is indicated by asterisks: * $p < 0.05$ (Student's *t* test).

2.4.8 Allelic effect, mother effect or father effect

To follow up, the indication that "affected" *Pts*-ki/ko mice only appeared when born from *Pts*-ki/ki or *Pts*-ki/wt females and not from *Pts*-ki/ki or *Pts*-ki/wt males suggested that the genotype (*Pts*-ki/ki or *Pts*-ki/wt) of the mother plays an important role.

Hence, we speculated whether the observed phenotypical diversity within the *Pts*-ki/ko mice was due to an allelic effect (homozygous versus heterozygous), a mother effect or a father effect (Figure 18).

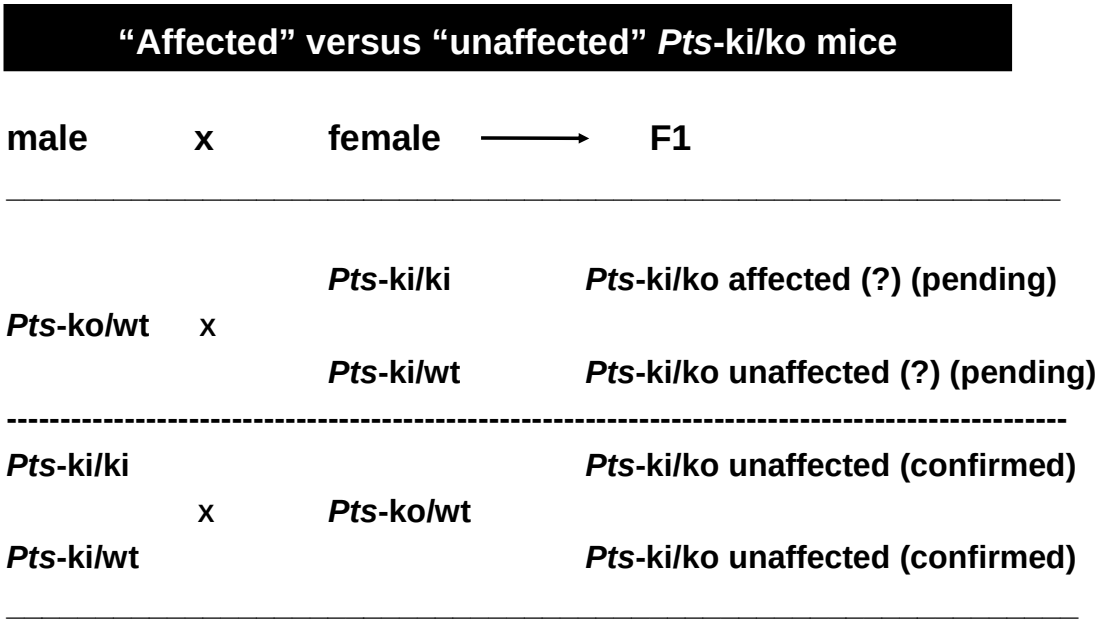

Figure 18: Hypothesis: Allelic effect (homozygous versus heterozygous), mother effect or father effect
The "affected" *Pts*-ki/ko mice were generated when *Pts*-ki/ki females or *Pts*-ki/wt females were bred with *Pts*-ko/wt males but not when *Pts*-ko/wt females were bred with *Pts*-ki/ki males or *Pts*-ki/wt males suggesting a tendency towards a *Pts*-R15C-ki mother effect. To test this hypothesis, we sat up the following breeding-pairs: 3x (*Pts*-ki/ki female and *Pts*-ko/wt male), 3x (*Pts*-ki/wt female and *Pts*-ko/wt male), 3x (*Pts*-ki/ki male and *Pts*-ko/wt female) and 3x (*Pts*-ki/wt male and *Pts*-ko/wt female).

To further investigate this hypothesis, we sat up the following breeding-pairs: a) *Pts*-ki/ki female and *Pts*-ko/wt male, b) *Pts*-ki/wt female and *Pts*-ko/wt male, c) *Pts*-ki/ki male and *Pts*-ko/wt female and d) *Pts*-ki/wt male and *Pts*-ko/wt female (Figure 18).

As in our previous breeding-studies, all the newborn mice were carefully monitored the first hours after birth. Diversity among the newborns were only observed within the mice born from *Pts*-ki/ki females (Figure 19) and the following day the mice that clearly stood out (undersized, passive, isolated/alone, cold and lack of milk (white-belly)) were either dead or eaten up.

Subsequently, mice from *Pts*-ki/ki females were sacrificed at day 1. Mice from the three other breedings-pairs (Figure 19) showed no apparent phenotype and survived until the age of three weeks where they were sacrificed. Additionally, newborn mice from *Pts*-ki/wt females, *Pts*-ki/ki males and *Pts*-ki/wt males were also sacrificed.

Currently, the tissue (brain and liver) and blood collected from the sacrificed mice (four breeding-pairs, five genotypes and two ages (newborn and 3 weeks of age (minus from *Pts*-ki/ki female and *Pts*-ko/wt)) are being analyzed in terms of PTPS enzyme activity (liver and brain), neopterin (liver and brain), biopterin (liver and brain), blood phenylalanine concentration and brain monoamine neurotransmitters. Moreover, in collaboration with Professor Hiroshi Ichinose and PhD. Daigo Homma, Tokyo Institute of Technology, Yokohama, Japan, whole brains from newborn have been collected to investigate the tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH) status by immunohistrochemistry.

Figure 19: Newborns from allelic effect, mother effect or father effect-breeding experiment
Significant diversity among the newborns were only observed within the mice born from *Pts*-ki/ki females (A) as they were undersized, passive, isolated/alone, cold and showed lack of milk-white-belly. If not sacrificed at day 1, these mice were either dead or eaten up the following day. A: Newborns from *Pts*-ki/ki female and *Pts*-ko/wt male. B: Newborns from *Pts*-ki/wt female and *Pts*-ko/wt male. C: Newborns from *Pts*-ki/ki male and *Pts*-ko/wt female and D: Newborns from *Pts*-ki/wt male and *Pts*-ko/wt female.

2.5 Discussion

In the present study, we generated two new *Pts* mouse models – a *Pts*-ki/ki mouse and a *Pts*-ki/ko mouse by targeting the biosynthetic *Pts* gene to study BH_4 deficiency, in particular the phenotypical diversities associated with human PTPS deficiency.

Our biochemical analysis revealed that the *Pts*-ki/ki mice neither have HPA nor considerable differences within the brain monoamine neurotransmitter metabolites compared to the *Pts*-ki/wt and wt/wt mice, but instead showed a significant reduced PTPS enzyme activity and elevated neopterin both in liver and brain. Despite, the phenotypic reduced PTPS enzyme activity and elevated neopterin, the *Pts*-ki/ki mice did not mimic human PTPS deficiency which is characterized by reduced PTPS enzyme activity, elevated neopterin, reduced biopterin, HPA and monoamine neurotransmitter deficiency in the CNS thus the *Pts*-ki/ki mouse is not a suitable mouse model to study BH_4 deficiency.

Although, the *Pts*-ki/ki mice manifested at phenotype too mild compared to the human BH_4 deficient situation, we sent them to the GMC for further comprehensive phenotypical and biochemical characterization to see whether the *Pts*-ki/ki allele would give rise to an unknown and unexpected phenotype. Intriguingly, the *Pts*-ki/ki mice did not revealed an unambiguous *Pts*-R15C-ki allele specific phenotype in terms of dysmorphology, behaviour, neurology, nociception, eye/vision, clinical chemistry, haematology, steroid metabolism, lung function or pathology. Yet, the comprehensive phenotypical and biochemical analysis demonstrated new and interesting aspects within energy metabolism and immunology/allergy which have to be investigated further on another cohort to confirm whether these observations are specific related to this specific cohort of mice or whether these observations are a general phenotype feature for the *Pts*-ki/ki mice.

In parallel with the comprehensive phenotypical and biochemical characterization at the GMC, we performed feeding-studies on *Pts*-ko/wt males and *Pts*-ki/ki males to confirm a previous unpublished observation suggesting that *Pts*-ko/wt males exhibit an abnormal fat distribution as they were shown to have a two-fold increase in intra-abdominal adipose tissue compared to a 1.6-fold increase in wt/wt males after subjected to high fat diet over a period of several weeks. Interestingly, we found that the *Pts*-ki/ki males, like the *Pts*-ko/wt males, displayed an abnormal fat distribution on both standard mouse chow and high fat diet.

This result not only supports the energy metabolism observations at the GMC but also demonstrates for the first time that insufficient PTPS enzyme activity plays an important role in the fat distribution. The underlying metabolic and/or molecular mechanisms linking PTPS deficiency with abdominal obesity remains unclear and has to be investigated further.

The most likely explanation for this linkage between insufficient PTPS enzyme activity and abdominal obesity is via endothelial nitric oxide synthase (eNOS). eNOS is under adequate BH_4 levels responsible for the NO production which plays an important role in the regulation of vascular tone by mediating endothelium-dependent vasodilatation (Schmidt and Alp, 2007). Moreover, NO is a potent inhibitor of platelet and leukocyte adhesion to the vascular wall and inhibits smooth muscle cell proliferation (Gesierich et al., 2003).

When BH_4 is limited the NO production is reduced and the superoxide (O_2^-) production by uncoupled eNOS is increased which causes vascular oxidative stress within endothelial cells (Schmidt and Alp, 2007). These endothelial imbalances have been associated with numerous vascular diseases including diabetes, hypertension, experimental vascular injury and hypercholesterolaemia (Schmidt and Alp, 2007, Werner et al., 2011).

Several studies have shown that endothelial dysfunction within e.g. streptozotocin-induced diabetic rats, hypercholesterolaemic ApoE-KO mice and Type 2 diabetic patients can be reversed by BH_4 (sepiapterin) supplementation (Ishii et al., 2001, Schmidt and Alp, 2007). Thus, BH_4 represents a potential therapeutic target for preserving eNOS function in vascular disease (Schmidt and Alp, 2007).

Seen in this prospective, it would be interesting to explore whether oral BH_4 administration would influence the abnormal fat distribution observed in the *Pts*-ko/wt and *Pts*-ki/ki males.

In addition, oxidative stress within endothelial cells has been shown to cause a reduction of BH_4 to BH_2 (Schmidt and Alp, 2007). For that reason, it could be interesting to measure the amount of BH_2 and/or the BH_4/BH_2 ratio in the *Pts*-ko/wt and *Pts*-ki/ki males after 10 weeks on standard mouse chow or high fat diet.

Beside the *Pts*-ki/ki mice, we also generated *Pts*-ki/ko mice by breeding the *Pts*-R15C-ki allele into the *Pts* null allele. Surprisingly, the genotyping results revealed two different phenotypes – “affected” *Pts*-ki/ko mice (dying 3-4 days after birth) and “unaffected” *Pts*-ki/ko mice (surviving until sacrificed at 3-4 weeks of age).

The underlying molecular mechanisms for the two phenotypes are unknown and will be investigated further.

Both the “affected” and “unaffected” *Pts*-ki/ko mice showed reduced PTPS enzyme activity in liver and brain as expected from *Pts*-ki/ki and homozygous *Pts*-ko mice. Unlike, the “unaffected” *Pts*-ki/ko mice, the “affected” *Pts*-ki/ko mice showed elevated neopterin levels (liver and brain), reduced biopterin levels (liver and brain), mild HPA and brain monoamine neurotransmitter deficiency due to undetectable levels of dopamine, HVA, serotonin and 5-HIAA. Interestingly, this phenotype imitates the human BH_4 deficient situation thus making the “affected” *Pts*-ki/ko mouse a suitable model to study human BH_4 deficiency.

In an attempt to elucidate, the phenotypical diversities observed among the *Pts*-ki/ko mice, newborn mice were sacrificed and biochemically characterized. The subsequent analysis revealed significant reduced PTPS enzyme activity (liver and brain), elevated liver neopterin and reduced brain biopterin levels but normal blood phenylalanine and monoamine neurotransmitter levels in the CNS compared to *Pts*-ki/wt, *Pts*-ko/wt and wt/wt mice. Thereby, demonstrating that “affected” and “unaffected” *Pts*-ki/ko mice set apart in terms of the liver biopterin, blood phenylalanine and brain monoamine neurotransmitters. This indication suggests that the phenotypical deviation observed between the “affected” and “unaffected” *Pts*-ki/ko occurs after birth.

In order to reproduce the “affected” *Pts*-ki/ko phenotype we breed *Pts*-ki/ki or *Pts*-ki/wt males with *Pts*-ko/wt females. This breeding experiment demonstrated that “affected” *Pts*-ki/ko mice only appeared when born from *Pts*-ki/ki or *Pts*-ki/wt females and not from *Pts*-ki/ki or *Pts*-ki/wt males suggested that the genotype of the parents plays an important role. Hence, we speculated whether the observed phenotypical diversity within the *Pts*-ki/ko mice was due to an allelic effect (homozygous versus heterozygous), a mother effect or a father effect.

Although still under investigation, our preliminary observations point toward a tendency of mother effect (*Pts*-ki/ki females) in combination with allelic effect (homozygosity) to be responsible for the diverse compound heterozygous phenotypes (Figure 19). The tendency of a homozygous mother effect is made based on observations indicating that “affected” *Pts*-ki/ko mice only arised from *Pts*-ki/ki females and not from *Pts*-ki/wt female, *Pts*-ki/ki males or *Pts*-ki/wt males (Figure 19).

We hypothesize that a transfer of biopterin from mother to the offsprings via the milk might be of functional significance and perhaps provide an explanation for the two diverse compound heterozygous phenotypes.

This hypothesis is supported by the observations indicating that the condition of the "affected" *Pts*-ki/ko mice becomes progressively more severe after birth when they depend on biopterin provided via the milk from their mothers. Another, indication in agreement of this hypothesis is the lack of milk-white-belly in the "affected" *Pts*-ki/ko mice.

Previous, biopterin measurements in different human body fluids and tissues have shown that the concentration of biopterin was highest in the milk (Matsubara and Gaull, 1985, Leeming et al., 1976, Dhondt et al., 1982). In fact, the biopterin concentration in milk was found to be 89 times higher than in serum which suggests that a dietary supply of biopterin might has an important role in the development of human infants (Matsubara and Gaull, 1985). Moreover, neopterin was also measured to be present in milk yet in smaller concentrations than biopterin (Matsubara and Gaull, 1985). Seen in this perspective, we would like to measure biopterin and neopterin concentrations in mother milk from lactating *Pts*-ki/ki females, *Pts*-ki/wt females, *Pts*-ko/wt females and wt/wt females using published mouse milking procedures (DePeters and Hovey, 2009, Ragueneau, 1986).

Altogether, the results presented in this study demonstrated that "affected" *Pts*-ki/ko mice is a suitable mouse model to study BH_4 deficiency as it mimics the biochemical hallmarks of human BH_4 deficiency thus can be used for further investigations of human BH_4 deficiencies and future treatment studies.

2.6 Supplementary Information

Primary screening at the GMC:

Anatomical observations and X-ray analysis showed no phenotypical differences between *Pts*-ki/ki and wt/wt mice but the bone densitometry showed significant decreased body mass content as well as increased body weight and lean mass in *Pts*-ki/ki males compared to wt/wt males. This observation was not seen in *Pts*-ki/ki females but only in *Pts*-ki/ki males thus a secondary dysmorphology screen was not suggested.

In terms of behaviour two tests were performed, an open field test and a pre-pulse inhibition test. The open field test revealed significant increased locomotor activity in the mutant mice compared to the control mice. However, no significant genotype effect was observed on the rearing activity (vertical locomotion) or centre time (anxiety-related behaviour). Moreover, no significant genotype effect was observed in the pre-pulse inhibition test.

Assessment and evaluation of basic neurological and motor functions were examined by a modified-SHIRPA protocol (systematical behavioural and functional observations). The SHIRPA-results revealed no difference in grip strength or rotarod but there was a tendency towards more activity/alertness (more active in viewing jar and showed more defecation) in the *Pts*-ki/ki mice compared to the wt/wt mice.

Nociception was investigated by exposing the mice to the hot plate test and the reaction-times indicated that no significant genotype or sex effect was found hence additional pain-related studies was not suggested. Examination of the eyes and vision by ophthalmoscope and slit lamp analysis showed no association of the *Pts*-R15C-ki allele and the occurrence of anterior segment abnormalities, retinal fundus abnormalities or axial eye length abnormalities thus no further tests were recommended.

Body composition analysis by non-invasive NMR to determine the body fat and lean mass content in the mice as well as the indirect calorimetry to determine the energy metabolism from gas exchange was performed. The NMR revealed that *Pts*-ki/ki mice have a significantly reduced body mass but in males the body fat content was increased and the lean mass was decreased (Figure 11). No shift in body composition could be detected in *Pts*-ki/ki females. No other variables monitored during the indirect calorimetry trial were substantially affected.

The spontaneous breathing (activity, rest and sleep) as a measure for the lung function showed a significant higher tidal volume in *Pts*-ki/ki females at activity resulting in higher values for body mass related parameters, yet no diversity in breathing pattern was found for

the *Pts*-ki/ki males. Besides this observation no genotype-related differences were observed in respiratory rates or timing (normal adaptation (respiratory rates) from rest to activity) between *Pts*-ki/ki and wt/wt mice.

Blood was collected by retroorbital puncture and subdivided for further analysis within the following areas: clinical chemical, haematology, allergy, immunology and steroids. Intraperitoneal glucose tolerance test showed no genotype-related difference but a tendency towards an increased plasma glucose levels was observed in fasted mutant mice. Moreover, the female mutant lost more weight than female control mice due to overnight fasting, conversely this genotype effect was not observed in males.

The clinical chemical parameters revealed a slightly increased (*Pts*-ki/ki females) and decreased (*Pts*-ki/ki males) sodium concentration, significant increased (*Pts*-ki/ki females) and decreased (*Pts*-ki/ki males) chloride concentration, increased cholesterol and triglyceride levels in both mutant males and females but mainly significant in *Pts*-ki/ki females. In addition, a higher alkaline phosphatase activity was observed in *Pts*-ki/ki mice compared to wt/wt mice.

The animal blood count showed at slightly increased haemoglobin concentrations in mutant animals resulting in significantly higher mean corpuscular haemoglobin and mean corpuscular haemoglobin concentration values.

The immunology and allergy screen showed no changes in the frequencies of most leukocyte subsets in peripheral blood but only subtle differences in the frequency of $CD11b^+$ expressing NK cells (a maturation marker for NK cells) and slightly lower levels of immunoglobulins IgG1, IgG2b and IgE in *Pts*-ki/ki female mice (Figure 12A). The immunoglobulin IgE concentration were re-measured in plasma samples from female mice (wt/wt and *Pts*-ki/ki) and also the second time the same tendency was observed (Figure 12B). Due to the lower levels of IgG1, IgG2b and IgE in *Pts*-ki/ki females a secondary test with a new cohort of mice (n=10 to 12 per sex per group) to confirm the observed phenotype was suggested.

Corticosterone showed a sex-specific difference within the wild-type mice conversely this was not observed in the *Pts*-ki/ki mice. In contrast, testosterone verified an expected sex-specific difference in both the *Pts*-ki/ki and wt/wt mice nevertheless no steroid phenotype was found in the *Pts*-ki/ki mice.

Finally, no pathological alternations were shown in the *Pts*-ki/ki mice compared to wt/wt mice upon dissection and macroscopic pathological examination as well as histological analysis.

2.7 References

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3

Targeting of murine *Gchfr* gene to study the role of GFRP *in vivo*

Running title: Conditional *Gchfr* knock-out mouse to study the role of GFRP *in vivo*

3 Targeting of murine *Gchfr* gene to study the role of GFRP *in vivo*

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3.1 Abstract

In the liver, BH_4 has been found to regulate its own biosynthesis through feedback inhibition of GTP cyclohydrolase I (GTPCH) mediated by GTP cyclohydrolase feedback regulatory protein (GFRP). Moreover, $_L$-phenyalanine has been shown to reverse this BH_4-mediated end-product feedback inhibition and instead imply a feedforward stimulation of the BH_4 biosynthesis.

In contrast to the liver, where phenylalanine stimulates the BH_4 biosynthesis by displacing BH_4 from the GTPCH-GFRP protein-complex, the regulation of BH_4 biosynthesis in the brain is not yet understood. However, GFRP seems to play an important role as studies in rat brain have shown that GFRP mRNA was abundant in serotonin neurons within the brainstem, but undetectable in dopamine neurons of the midbrain or norepinephrine neurons of the locus coeruleus. Moreover, analysis in primary neuronal cultures have indicated that GFRP regulates the BH_4 biosynthesis in serotonin neurons but not in dopamine or norephinephrine neurons which suggest that GFRP has a specific function in brain serotonin homeostasis.

To study the *in vivo* role of GFRP in liver and brain as well as its potential contribution to human diseases, we aimed of generating the first animal model for GFRP, a conditional knock-out by targeted mutagenesis of the murine *Gchfr* gene. Besides understanding the *in vivo* function of the *Gchfr* gene by studying the consequences of the *Gchfr* knock-out, we aim to find clinical relevant phenotype in the GFRP-deficient mice and thereby identify a human-specific counterpart.

3.2 Introduction

BH_4 is synthesized *de novo* from guanosine triphosphate (GTP) by three enzymes: GTP cyclohydrolase I (GTPCH), 6-pyruvoyl-tetrahydropterin synthase (PTPS) and sepiapterin reductase (SR) (Thöny et al., 2000) (Figure 1). The committing step of the BH_4 biosynthesis is the conversion of GTP to NH_2TP catalyzed by GTPCH (Thöny et al., 2000). The GTPCH activity, which directly affects the BH_4 levels, is predominantly regulated at the transcriptional level by various proinflammatory cytokines such as interferon-γ, interleukin 1β and tumor necrosis factor-α as well as bacterial lipopolysaccharide but is also regulated at the post-transcriptional level by phosphorylation (Gesierich et al., 2003, Milstien et al., 1996, Werner-Felmayer et al., 2002, Imazumi et al., 1994, Lapize et al., 1998). Additionally, *in vitro* studies on rat liver extracts have demonstrated that BH_4 regulates its own biosynthesis through feedback inhibition of GTPCH and that this end-product feedback inhibition is mediated through protein-complex formation with GTP cyclohydrolase feedback regulatory protein (GFRP) (Harada et al., 1993). Furthermore, $_L$-phenylalanine has been shown to reverse this BH_4-mediated feedback inhibition as it changes the inactive GTPCH-GFRP complex to its active form (Yoneyama et al., 1997).

Whereas, the BH_4 biosynthesis in the liver is well understood, the regulation of BH_4 biosynthesis in brain is not yet known. Even so, GFRP appears to play an important role as primary neuronal culture studies have demonstrated that GFRP regulates BH_4 biosynthesis within serotonin neurons but not within dopamine or norephinephrine neurons suggesting that GFRP is specifically expressed in some brain tissue or cell types (Kapatos et al., 1999).

Based on these indications, we hypothesize that GFRP has a specific function in brain serotonin homeostasis. To study the *in vivo* role of GFRP in liver and brain in further details as well as its potential contribution to human s, we aim to generate the first animal model for GFRP, a conditional knock-out for the murine *Gchfr* gene.

When the conditional *Gchfr* knock-out mouse has been successfully generated, we attend to generate mice with a complete, a peripheral (liver-specific and kidney-specific) and a neuronal *Gchfr*-deletion (CNS-specific and brain-tissue-specific) and subsequently subject them to comprehensive phenotypical analysis including biochemical and metabolic investigations on BH_4-and neurotransmitter-related pathways. In that way learn why GTPCH is regulated by BH_4 and phenylalanine via GFRP in some cell types but not in others. In the study presented here, we describe the two *Gchfr* gene targeting strategies used in order to generate the first animal model for GFRP, a conditional knock-out for the murine *Gchfr* gene.

3.3 Method and materials

Gene targeting strategy

The murine *Gchfr* gene (Ensembl Transcript ID: ENSMUSG00000046814) encoding for GFRP is located at chromosome 2 and consists of 3 exons: 101 bp, 95 bp and 449 bp, respectively. In order to generate a conditional *Gchfr* knock-out mouse a targeting vector leading to deletion of exon 2 was cloned (PolyGene Transgenetics AG, Switzerland). Deletion of exon 2 was chosen as a conditional insertion 5' of exon 1 would pose a risk of interfering with the poorly characterized promoter region. On the other hand, involvement of exon 3 would likely interfere with expression of a mapped gene in close proximity in opposite direction and with unknown function. The crystal structure of GFRP (Maita et al., 2002) suggested that a deletion of exon 2 would cause inhibition of the two binding sites for GTPCH and phenylalanine, respectively, leading to a total loss of function hence at targeting vector with a "floxed" exon 2 was generated.

Construction of D153 targeting vector

Targeting vector D153 was constructed with a 1282 bp short arm of homology and a 5.3 kb long arm of homology including exon 2 and 3 of the *Gchfr* gene. In addition, a pseudo-vector D152 (not shown) was generated for setting up the screening conditions of the transfected ES cell clones. For positive (neomycin gene) and negative (thymidine kinase (TK) gene) selection a LoxP-neo-TK-LoxP cassette was introduced between the short and the long arm of homology. Moreover, a LoxP site was added 200 bp upstream of exon 2. The targeting vector, which is shown in Figure 20A, was after successful construction transfected into ES cells derived from the C57BL/6 strain. Positive ES cell clones, in which homologous recombination has occurred, were genetic characterized by PCR (not shown) as well as Southern blot analysis using external 5' and 3' probes (not shown). Both the PCR and Southern blot analysis were performed by Dr. Tanja Scherer and M.Sc. David Meili.

Several studies have revealed that TK expressing mice are infertile (Braun et al., 1990) thus the LoxP-neo-TK-LoxP cassette was deleted by transfecting one correctly targeted ES cell clone with Cre recombinase. Cre recombinase transfection led to three different targeted *Gchfr* loci depending on the recombination between the three LoxP-sites (Figure 20B). Correct Cre recombination event was determined by Dr. Tanja Scherer using a PCR approach (not shown).

One ES cell clone with “floxed” exon 2 was chosen for morula aggregation which was carried out at the Institute of Laboratory Animal Science at the University of Zürich in collaboration with Dr. Thorsten Buch and Dr. Pawel Pelczar (Figure 20B). Morula aggregation led to generation of four chimeras which were subsequently back-crossed with mice derived from the C57BL/6-albino strain (B6-Tyr <cBrd> provided by Dr. Pawel Pelczar at the Institute of Laboratory Animal Science) in order to generate heterozygous *Gchfr*-floxed mice (Figure 21).

Alternative strategy: Construction of D154 targeting vector

A 2048 bp fragment including a LoxP-FRT-neomycin-FRT cassette from the BBV16rapidflirt vector (kindly provided by Dr. Thorsten Buch) was subcloned into the D151 targeting vector (previous used to generate targeting vector D153 and pseudo-vector D152) consisting of exon 2 of the murine *Gchfr* gene and a LoxP-site 200 bp upstream of exon 2. The subcloning which was performed by TOPO Gene Tech led to the generation of a LoxP-FRT-neo-FRT-exon2-loxP targeting vector called D154 (Figure 22). Recently, this targeting vector was recently linearized and electoporated into ES cells derived from the C57BL/6 strain.

3.4 Results

Targeted disruption of the Gchfr gene

In order to generate a conditional knock-out mouse of the *Gchfr* gene encoding for GFRP, we chose a classical strategy for homologous recombination in C57BL/6 derived embryonic stem (ES) cells using a targeting vector (D153) containing a LoxP-neo-TK-LoxP-exon2-LoxP motif to "flox" exon 2 of the murine *Gchfr* gene and thereby generate a conditional *Gchfr* knock-out mouse. The targeting strategy for deletion of the murine *Gchfr* gene is depicted in Figure 20.

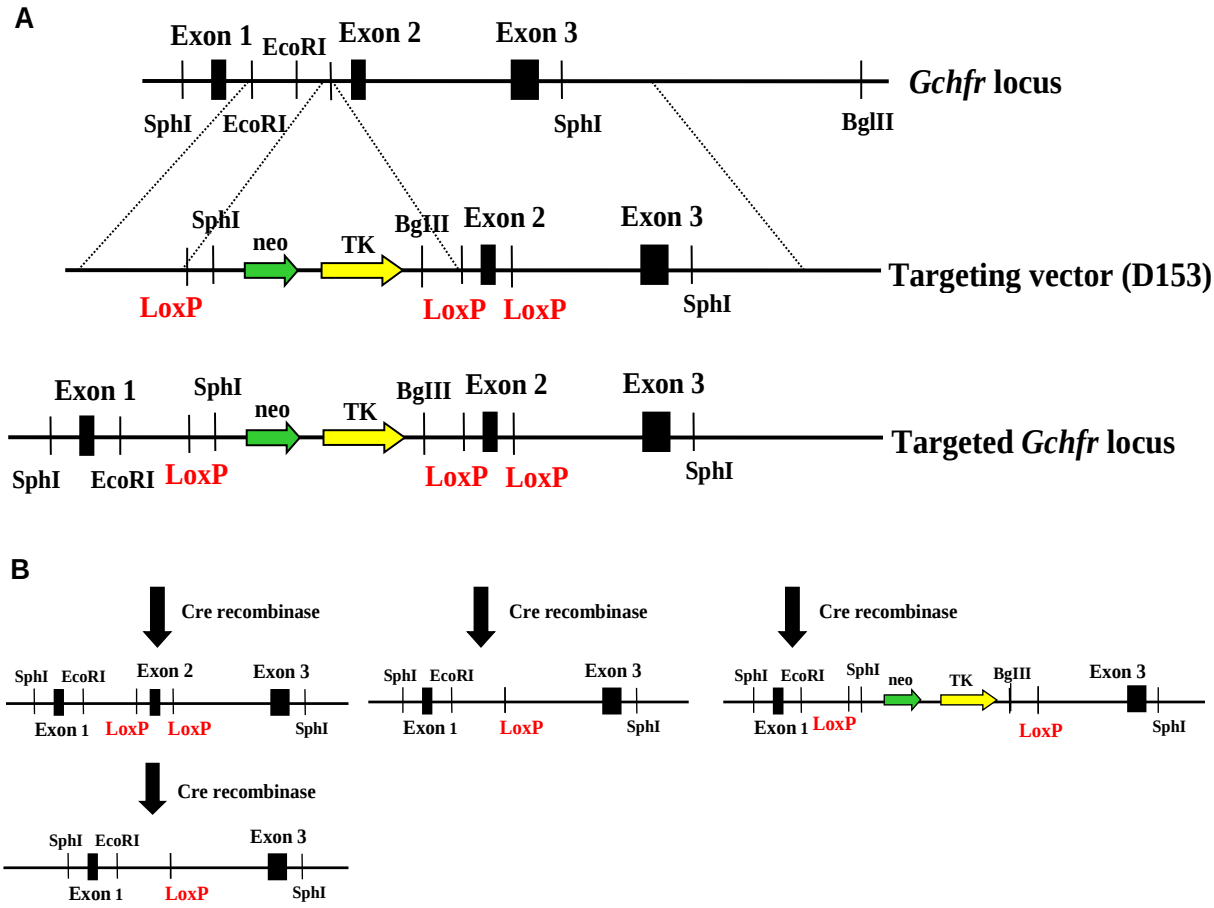

Figure 20: Targeting vector D153 used to generate conditional *Gchfr* knock-out mice

A: Schematic representation of *Gchfr* gene locus (top), targeting vector D153 including a LoxP-neo-TK-LoxP-cassette (middle) and the *Gchfr* gene locus after the homologous recombination event (bottom). B: Transfection of the ES cells with Cre recombinase in order to delete the neo-TK-cassette cause three different targeted *Gchfr* loci depending on the Cre recombination event between the LoxP-sites. Deletion of the neo-TK-cassette is necessary as studies have shown that TK expressing mice are infertile. The "floxed" exon 2 locus (left) result in a conditional *Gchfr* knock-out mouse which requires breeding with a Cre-deleter mouse (complete, tissue-specific or cell-specific) to generate a complete *Gchfr* knock-out mouse. Both the LoxP (middle) and LoxP-neo-TK-LoxP (right) targeted *Gchfr* loci result in complete *Gchfr* knock-out mice (not conditional).

After successful construction of targeting vector D153 and correctly targeting into C57BL/6 derived ES cells, followed by morula aggregation, four chimeras were born (Figure 21).

The four chimeras (3 males and 1 female, one male was ~ 5% chimeric but the three others were 50-80 % chimeric) were at the sexual mature age back-crossed with mice derived from the C57BL/6-albino strain to generate heterozygous *Gchfr*-floxed mutant mice (Figure 21).

Figure 21: Two chimeras born after morula aggreation together with three C57BL/6-albino littermates
Morula aggregation at the Institute of Laboratory Animal Science at the University of Zürich in collaboration with Dr. Thorsten Buch and Dr. Pawel Pelczar led to generation of four chimeras (3 males and 1 female, one male was ~ 5% chimeric but the three others were 50-80 % chimeric). In this figure, two of the four chimeras born after morula aggregation (both in left corner with black (C57BL/6)/white (C57BL/6-albino) fur color) are depicted together with three of their C57BL/6-albino (all with white fur and red eyes) littermates.

Regardless of the relative high chimerism, the four chimeras did not cause generation of heterozygous *Gchfr*-floxed mice indicating lack of germline transmission. Hence, the morula aggregation step was repeated to achieve new chimeric mice with perhaps an even higher chimerism. Despite, using several different and improved morula aggregation strategies, the correctly *Gchfr* targeted ES cell clones did not led to generate of new chimeras as consequence a second and new targeting vector, named D154, was generated (Figure 22). Altogether, this alternative *Gchfr* gene targeting strategy involving an improved targeting vector D154 and new ES cells was decided as the previous correctly D153 targeted ES cell clones were shown to be unworkable for the generation of chimeric mice.

The second and alternative gene targeting strategy for generation of conditional *Gchfr* knock-out mice using a newly generated targeting vector D154 is depicted in Figure 22.

In contrast to the previous targeting vector D153, the new and improved targeting vector D154 consisted of "only" two LoxP-sites surrounding the exon 2 (this was done in order to decrease the number of recombination possibilities between the LoxP-sites after Cre recombinase transfection). Moreover, targeting vector D153 contained a FRT flanked neomycin-cassette between the two LoxP-sites for positive selection of the ES cells (Figure 22). Construction of the D154 targeting vector was successful thus it was recently linearized and electoporated into ES cells derived from the C57BL/6 strain. At this current state, we are ready to analyze positive *Gchfr* targeted ES cell clones by PCR and Southern blot analysis (same screening strategy as used for targeting vector D153) to confirm correctly insertion of targeting vector D154 after homologous recombination in the ES cells.

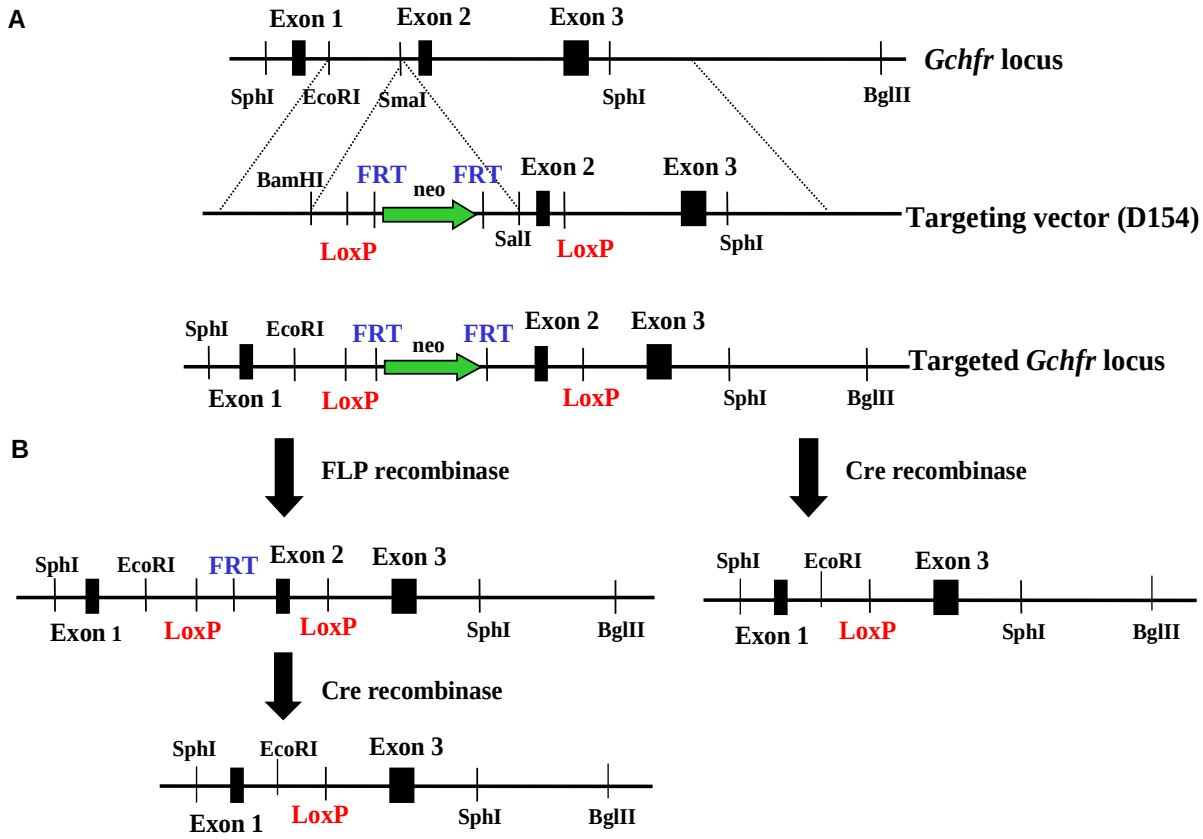

Figure 22: Alternative targeting strategy to generate conditional *Gchfr* knock-out mice

A: Schematic representation of *Gchfr* gene locus (top), targeting vector D154 including the LoxP-FRT-neo-FRT-cassette (middle) and the *Gchfr* gene locus after the homologous recombination where the genomic DNA has been introduced into the ES cells (bottom). B: Targeting vector D154 contains two LoxP-sites and a flanked neo-cassette which means that breeding with a FLP recombinase and/or Cre recombinase expressing mice will lead to the generation of a *Gchfr* knock-out mouse.

3.5 Discussion

This present study, describes two different gene targeting strategies used for the generation of the first animal model for GFRP, a conditional knock-out for the murine *Gchfr* gene.

The initial gene targeting strategy which was based on a targeting vector (D153) consisting of a LoxP-neo-TK-LoxP-exon2-LoxP motif, led via morula aggregation to the generation of four chimeras (three males and one female, one male was ~ 5% chimeric but the three others were 50-80 % chimeric) (Figure 21).

Despite the relative high chimerism of the four chimeras no heterozygous *Gchfr*-floxed mice were generated after back-crossing with C57BL/6-albino mice indicating the lack of germline transmission. Furthermore, replication of the morula aggregation step in order to generate new chimeric mice using the same ES cell clone were unsuccessful suggesting that the ES cells no longer were useful for generation of chimeras. This last observation was presumably due to the additionally step involving transfection with cre recombinase in order to delete the TK-cassette which in previous studies have been shown to cause infertile mice (Braun et al., 1990). Due to lack of germline transmission and the overall condition of the ES cells, a second *Gchfr* gene targeting strategy was launced.

Recently, the new and improved targeting vector (D154), which consists of a LoxP-FRT-neomycin-FRT-exon2-loxP motif has been linearized and electoporated into ES cells derived from the C57BL/6 strain thus we are now ready to genetic characterize the correctly transfected ES cell clones by PCR and Southern blot analysis.

Assuming that the *Gchfr*-knock-out mouse after successful generation is not exhibit lethal, we aim to generate and characterize different tissue-specific and neuron-specific GFRP knock-out mice including a complete *Gchfr* knock-out mouse, a peripheral *Gchfr* knock-out mouse (liver-specific and kidney-specific) and a neuronal *Gchfr* knock-out mouse (both an entire *Gchfr* brain deletion (CNS-specific) and brain tissue-specific).

At this state, we hypothesize that a complete deletion of the *Gchfr* gene is not essential but rather is associated with dystonia or potentially developmental abnormalities. Nevertheless, comprehensive phenotypical, behavioral and molecular analysis of the *Gchfr* mouse models will assess this hypothesis, but also facilitate the searching for a human counterpart. As GFRP is the only gene within the BH_4 metabolism for which no human deficiency has yet been described (Thöny and Blau, 2006).

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4

Autism associated with low 5-HIAA in CSF and the heterozygous *SLC6A4* gene Gly56Ala plus 5-HTTLPR L/L promoter variants

Running title: Autism associated with low 5-HIAA in CSF and *SLC6A4* gene variants

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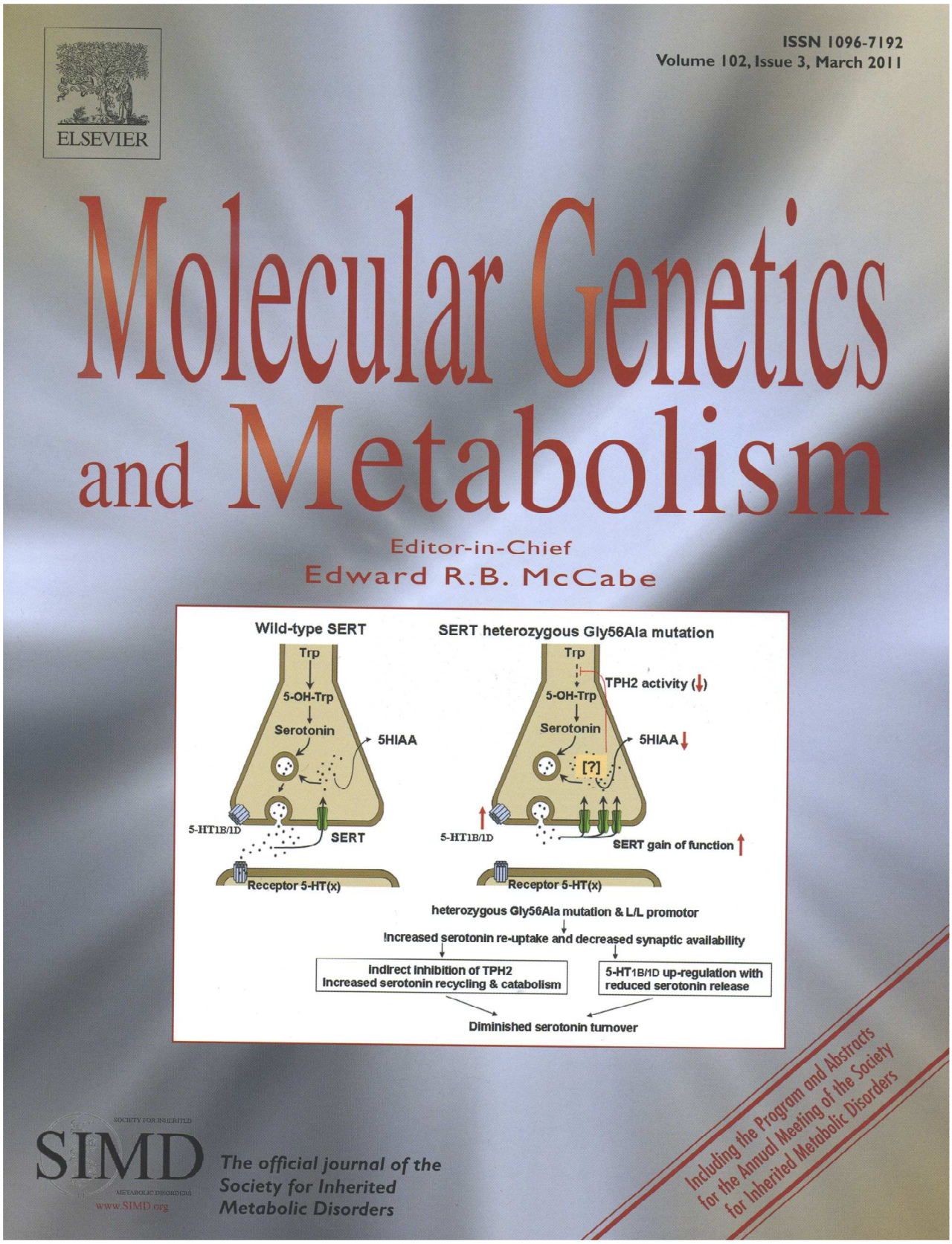
4 Autism associated with low 5-HIAA in CSF and the heterozygous *SLC6A4* gene Gly56Ala plus 5-HTTLPR L/L promoter variants



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Autism associated with low 5-hydroxyindolacetic acid in CSF and the heterozygous *SLC6A4* gene Gly56Ala plus 5-HTTLPR L/L promoter variants

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ABSTRACT

The known Gly56Ala mutation in the serotonin transporter SERT (or 5-HTT), encoded by the *SLC6A4* gene, causes increased serotonin reuptake and has been associated with autism and rigid–compulsive behavior. We report a patient with macrocephaly from birth, followed by hypotonia, developmental delay, ataxia and a diagnosis of atypical autism (PDD-NOS) in retrospect at the age of 4½ years. Low levels of the serotonin end-metabolite 5-hydroxyindolacetic acid (5HIAA) in CSF were detected, and *SLC6A4* gene analysis revealed the heterozygous Gly56Ala alteration and the homozygous 5-HTTLPR L/L promoter variant. These changes are reported to be responsible for elevated SERT activity and expression, suggesting that these alterations were responsible in our patient for low serotonin turnover in the central nervous system (CNS). Daily treatment with 5-hydroxytryptophan (and carbidopa) led to clinical improvement and normalization of 5HIAA, implying that brain serotonin turnover normalized. We speculate that the mutated 56Ala SERT transporter with elevated expression and basal activity for serotonin re-uptake is accompanied with serotonin accumulation within pre-synaptic axons and their vesicles in the CNS, resulting in a steady-state of lowered serotonin turnover and degradation by monoamine-oxidase (MAO) enzymes in pre-synaptic or neighboring cells.

1. Introduction

Autism is a childhood-onset disorder with neuro-developmental abnormalities in early life leading to severe defects in social interaction, communication and limited activities with repetitive stereotypic behavior or movements [1]. The Autism Spectrum Disorders (ASD) include besides the "typical" Kanner autism, Asperger's syndrome and the atypical forms of autism, also known as pervasive developmental disorder — not otherwise specified (PDD-NOS). In more than 25–50% of individuals suffering from ASD and their first-degree relatives, whole blood serotonin levels were found to be elevated due to increased serotonin storage within platelets [2,3]. In addition, functional neuro-imaging studies using positron emission tomography (PET) have shown diminished serotonin synthesis in children with autism between the age of 2 and 5 years, as well as the focal aberrations of serotonin synthesis and the low binding potentials of the serotonin transporter and receptor in individuals with ASD [4,5]. Short-term dietary depletion of tryptophan (i.e. the serotonin precursor) has been shown to exacerbate repetitive behavior and to elevate anxiety and feelings of unhappiness in autistic individuals [6]. In contrast, treatment with selective serotonin re-uptake inhibitors – commonly used antidepressants – interacts pharmacologically with the serotonin transporter SERT (or 5-HTT) and has been shown to be effective in ameliorating the repetitive and/or obsessive behavior and interests in some but not all autistic individuals [7].

The serotonin transporter SERT or 5-HTT, encoded by the *SLC6A4* gene, is thought to play a prominent role in serotonin homeostasis in primates and non-human primates, and several gene variants that may change the structure or function of the transporter protein were associated with autism [8]. Among them, polymorphisms within the 5-HTTLPR promoter sequence, mutations in the coding sequence, or intronic mutations of this serotonin transporter were reported to be linked to autism by some studies, but other studies have been inconclusive [8–10]. The *SLC6A4* 5-HTTLPR promoter sequence, located about 1 kb upstream of the transcription initiation site, contains two variable repeat length polymorphisms known as the long (L) with 16 repeat elements, or the 44 bp shorter (S) variant with 14 repeat

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elements [11,12], which determines a different expression of the serotonin transporter in pre-synaptic axonic membranes. The L/L genotype of this 5-HTTLPR promoter variant expresses significantly (1.4–1.5 fold) more transporter protein as compared to the L/S or S/S variants [13]. As a consequence, the L/L variant was proposed to lead to a lowered concentration of serotonin in the synaptic cleft, although contradictory reports regarding the role of 5-HTTLPR variations exist (see e.g. [14]). In addition to these non-coding variants in the promoter region, a number of mutations in the coding region modulate SERT activity [10]. Among them, the non-synonymous variant Gly56Ala in exon 3 of the *SLC6A4* gene was reported to be autism-associated and showed constitutively elevated SERT activity [15]. This glycine to alanine exchange at position 56 is located in the N-terminal intracellular tail of the SERT-protein. Pharmacological studies of serotonin re-uptake by EBV-transformed lymphocytes expressing the heterozygous Gly56Ala or homozygous 56Ala protein mutants indicated a progressive gain of function by 1.4 or 1.8 fold, respectively, compared to cells expressing the wild-type SERT (Gly56) [15]. Another known variant of the *SLC6A4* gene, which potentially modulates serotonin transport function, is a variable number of tandem repeat (VNTR) polymorphism in intron 2, STin2.9, STin2.10, and STin2.12, containing 9, 10 and 12 copies of the VNTR element, respectively [8,16].

The *in vitro* findings of increased serotonin re-uptake by the heterozygous SERT mutation 56Ala by pre-synaptic axons will be expected to enhance both serotonin recycling into vesicles and degradation by monoamine-oxidase (MAO) enzymes to the 5HIAA end-metabolite. 5HIAA measurements in CSF are thought to provide an accurate and comparable parameter for extra-cellular brain serotonin levels [17]. However, the adaptive changes with respect to serotonin autoreceptor expression, release of serotonin and its *de novo* synthesis remain unknown. Our report on a patient with atypical autism and speech delay carrying the heterozygous Gly56Ala allele of the *SLC6A4* gene in combination with the 5-HTTLPR L/L promoter variant documents for the first time findings of low 5HIAA in CSF and response to therapeutic intervention with 5-hydroxytryptophan/carbidopa with clinical improvement and normalization of 5HIAA in CSF, indicative of normal serotonin turnover in the brain.

2. Subjects and methods

2.1. Case report

This patient described here was reported in a previous paper (patient CM; number 3; [18]) as a boy from healthy, non-consanguineous parents, born after an uneventful prenatal period with a birth weight of 3450 g, 52 cm body length and 38 cm head circumference. After a normal neonatal period, the boy was described as a floppy infant during the first months, and his history revealed macrocephaly at and from birth with hypotonia and moderate delay of acquisition of motor milestones. He was able to pull himself to stand at 20 months and started to walk with an ataxic gait from the age of 2½ years. His development was marked by psychomotor retardation with speech delay and development of a hypotonic–ataxic syndrome at the age of 3½ years. In retrospect, a diagnosis of atypical autism at the age of 4½ years was suspected and could be confirmed recently as a review of the patient's history. The patient fulfilled the DSM-IV criteria for atypical autism (or PDD-NOS), being at that time all criteria of qualitative impairment of social interaction (4 items), all criteria for qualitative impairment in verbal and non-verbal communication (4 items), but lack of the criteria for restricted repetitive and stereotyped patterns of behavior, interests and activities, as manifested by 4 items. Before the age of 4½ years, he also had expressive speech delay as mentioned and did not show symbolic or imaginative play with severely disrupted social interaction and his condition could not be better accounted for by other neurological disorders. In summary, these manifestations comply with atypical autism at the age of 4½ years. Other testing like ADOS, ADI-R or CARS has not been performed at that time.

As mentioned before, the patient has been reported in a previous paper (patient number 3; [18]), describing five boys suffering from a novel neuro-developmental condition with psychomotor delay and a hypotonic–ataxic syndrome. All five patients had isolated low 5HIAA levels in CSF and responded to treatment with the serotonin precursor 5-hydroxytryptophan and the peripheral decarboxylase inhibitor carbidopa. Side-effects have never been reported. Following treatment with 5-hydroxytryptophan from the age of 4½ years, the here presented patient started to improve his motor skills and gait, fell less frequently and became able to use stairways. Much of improvement of his motor skills related to diminution of ataxic signs and better muscle tone. About 4 months after treatment the parents noticed that he started to utter sounds and attempted to speak single words and tried to add gestures to communicate to his brother and parents. From the age of 5½ years carbidopa was added to 5-hydroxytryptophan. He soon started to speak and communicate with interest in social interaction with his peers and other persons. The parents described as if he awakened from his own world. At the age of 5.9 years he had still an expressive language disorder and a non-verbal IQ test estimated his IQ between 80 and 90 [19]. The now nearly 17 year-old boy continued to receive treatment of 5-hydroxytryptophan and carbidopa from the age of 4 ½ years.

Although a central disorder of serotonin synthesis or low serotonin turnover was suspected, further genetic analysis of the genes encoding the neuronal and peripheral tryptophan hydroxylase genes was normal. Extensive investigations were normal, including neurophysiological studies, neuro-imaging, metabolic and genetic testing.

2.2. Methods

Lumbar puncture was performed according to a fixed protocol between 8:30 and 10:00 a.m. as described previously for determination of the biogenic amine metabolites, pterins and 5-methyltetrahydrofolate [20,21]. A fasting amino-acid analysis in plasma and CSF was performed on the patient.

After parental informed consent for the boy, an oral dose of 5-hydroxytryptophan was started. At the age of 4½ years, the child was put on a normal diet, excluding tryptophan rich nutrients, and 24-hour urinary 5HIAA excretion was measured before and after a low testing dose of 5-hydroxytryptophan at 1 mg/kg/day (tid) after hospital admission. Simultaneously, clinical tolerability during his stay in hospital was tested using a regular check-up of his general and neurological status as well as continuous monitoring of cardiopulmonary and circulatory parameters. Thereupon, oral treatment by 5-hydroxytryptophan was slowly increased at biweekly intervals with increments of 0.5 to 1 mg/kg/day (tid), up to a maximum daily dose of 7 mg/kg (tid) with regular verification of his clinical response and adverse side-effects. During treatment at this maximum 5-hydroxytryptophan dose, a lumbar puncture was repeated at the age of 5.2 years to check his spinal fluid 5HIAA levels. As spinal fluid 5HIAA and 5-hydroxytryptophan levels had remained low, we decided to reduce the 5-hydroxytryptophan dose by 30% to 5 mg/kg/day (tid) and added a low dose of carbidopa at 1 mg/kg/day (tid) from the age of 5½ years. During combined treatment with 5-hydroxytryptophan and carbidopa a CSF sample was repeated at the age of 5.9 years.

Genetic analysis included genomic DNA sequence analysis from various genes (see below). For the *SLC6A4* gene, we additionally investigated the 5-HTTLPR promoter variants and the intronic (intron 2) STin2 VNTR polymorphisms. All exons and exon–intron boundaries were sequenced from the following genes: tryptophan hydroxylases type 1 and 2 (*TPH1*, MIM# 191060, ENST0000250018; *TPH2*, MIM# 607478, ENST00000333850), GTP cyclohydrolase I feedback regulatory protein (*GCHFR*, MIM# 602437, ENST00000260447) and serotonin transporter (*SLC6A4*, MIM# 182138, ENST00000394821). More detailed

information on the molecular analysis of the *SLC6A4* gene, including the 5-HTTLPR and STin2 VNTR, is given in "Supporting information" and in Table S1.

3. Results

The CSF findings for our patient are depicted in Table 1 (see also reference [18]). The only aberrant baseline value for the metabolites analyzed was the lowered 5HIAA value in the presence of normal 5-hydroxytryptophan and tryptophan levels. The values for the catecholamine pathway metabolites L-dopa, 3-O-methyldopa and homovanillic acid (HVA), as well as the pterin metabolites (biopterin, neopterin) and 5-methyltetrahydrofolate were normal (not shown). The low 5HIAA value explained the elevated HVA/5HIAA ratio. A twenty-four hours urinary collection on a diet excluding food products with a high tryptophan content detected a low daily 5HIAA urinary excretion of 5 μmol (reference range 10–50 μmol/day), which normalized to a normal daily excretion of 71 μmol after an oral load of 5-hydroxytryptophan at 1 mg/kg body weight/day (divided in three daily doses).

A slowly titrating oral dose in the patient was started up to a final daily dose of 7 mg/kg 5-hydroxytryptophan, and from the age of 5½ years we reduced 5-hydroxytryptophan to 5 mg/kg and added 1 mg/kg carbidopa, divided into three equal doses. Clinical recovery resulted first in disappearance of hypotonia and ataxia, later followed by slow disappearance of autistic features and amelioration of speech and cognitive skills. However, speech delay persisted. Follow-up CSF analysis during oral substitution with 5-hydroxytryptophan alone (7 mg/kg/day) did not show normalization of CSF 5HIAA but addition of carbidopa showed normalization of 5HIAA values and the HVA/5HIAA ratio (see Table 1).

The isolated low serotonin metabolite 5HIAA in the CSF prompted us to investigate by a candidate gene approach factors that might be specifically involved in the metabolism or transport of serotonin. Among them, we tested for alterations in the *TPH1* and *2* genes, as well as the gene encoding the GTP-cyclohydrolase I feedback regulatory protein (GFRP, *GCHFR* gene). Whereas both tetrahydrobiopterin-dependent TPH isoforms are rate limiting enzymes for serotonin biosynthesis [22,23], the GFRP was reported to be expressed in specific brain tissues and potentially be involved in the regulation of tetrahydrobiopterin biosynthesis in serotonin (but not dopamine) neurons [24]. Only wild-type sequences were found for the *TPH1/2* and *GCHFR* genes (not shown).

Upon sequencing the coding exons plus exon–intron boundaries of the SERT transporter *SLC6A4* gene, we found in exon 3 at amino-acid codon 56 the heterozygous nucleotide variant c.167G>C, leading to a glycine to alanine exchange (also known as rs6355; see Fig. 1). The heterozygous *SLC6A4* Gly56Ala allele was inherited from his mother. Besides this well known mutant allele, we should mention that additionally we found a homozygous SNP in the non-coding exon 2, c.−185C>A (rs6354).

The *SLC6A4* gene promoter sequence 5-HTTLPR showed the homozygous L/L variant for the patient, whereas his mother had the L/S and the father had the L/L variants (Fig. 2A). As indicated in the Introduction, the L/L variant should lead to a lower serotonin concentration in the synaptic cleft than the other two variants [13].

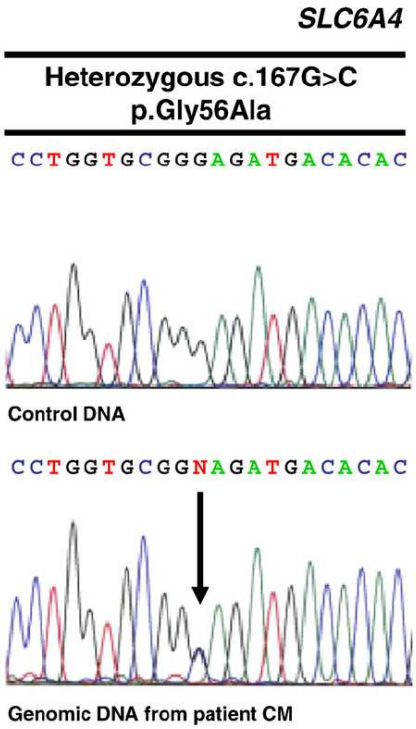

Fig. 1. Detection of the heterozygous variant c.167G>C (p.Gly56Ala) in exon 3 of the *SLC6A4* gene in patient CM with atypical autism. For more details see Methods plus *Supporting information* and *Table S1*.

As a final genetic alteration potentially influencing the activity of SERT, we analyzed the STin2 VNTR region in intron 2, and found the 9/10 variant for the patient, 10/12 for his mother and 9/10 for his father (Fig. 2B).

The mutations and variants found in the *SLC6A4* gene in this family are summarized in Table 2. In summary, a patient with atypical autism was found to carry the heterozygous Gly56Ala allele in the *SLC6A4* gene and exhibited low 5HIAA in CSF. Clinical improvement and normalization of serotonin turnover in the brain was found upon therapeutic intervention with 5-hydroxytryptophan/carbidopa. The implications of these findings will be discussed below.

4. Discussion

Allelic heterogeneity at the serotonin transporter gene locus (*SLC6A4*) was reported to confer susceptibility to autism and rigid–compulsive behavior. The patient we report here has a history of impaired motor and speech development *and* atypical autism or PDD-NOS. Moreover, we found isolated low 5HIAA in CSF indicative for low extracellular serotonin levels in brain. As documented in previous

Table 1
CSF findings in patient CM with the heterozygous *SLC6A4* Gly56Ala mutation.

Patient	Age (yrs)	5HIAA (nmol/l)	HVA (nmol/l)	HVA/5HIAA	Remarks
CM	3.7	102	372	3.6	No treatment
	5.2	107	334	3.1	7 mg/kg/day 5-hydroxytryptophan
	5.9	186	367	2.0	5 mg/kg/day 5-hydroxytryptophan and 1 mg/kg/day carbidopa
Ref.	2–4	202 (105–299)	603 (211–871)	1.5–3.5	
	5–10	133 (88–178)	523 (144–801)	1.5–3.5	

Reference values and ranges see also [21].

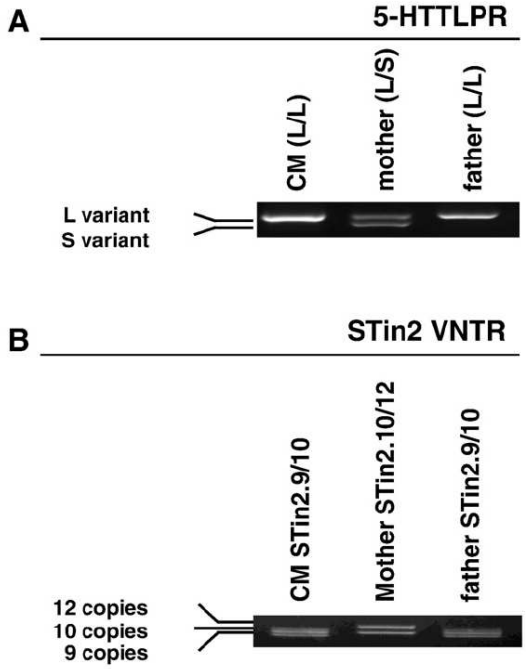

Fig. 2. *SLC6A4* 5-HTTLPR promoter and gene variants. (A) Amplification of 5-HTTLPR promoter variants. The PCR products at 523 bp and 479 bp indicate the present of the L (long) variant and the S (short) variant with a 44 bp deletion, respectively. (B) Analysis of the STin2 VNTR variants by PCR from patient CM and his parents. Amplification of the VNTR region in intron 2 (STin2) reveals the number of repetitive elements: 345 bp (9 copies), 360 bp (10 copies) or 390 bp (12 copies).

studies on autism [17], the presence of the Gly56Ala *SLC6A4* mutation would be expected to lead to increased uptake and a serotonin overload within platelets, where the protein mutant is expressed. Furthermore, at the brain level, the consequent intracellular serotonin overload among SERT Gly56Ala heterozygous or homozygous subjects would be expected to increase serotonin re-uptake into pre-synaptic vesicles. Serotonin accumulation within pre-synaptic cells (or neighboring neurons and/or astrocytes) might predispose to secondary diminished serotonin metabolism and degradation by MAO enzymes. Evidence from functional neuro-imaging studies in autism has indicated low serotonin production within the brain between 2 and 5 years, while dietary tryptophan depletion aggravated the autistic behavior. However, the exact mechanism underlying these observations of diminished serotonin production in the brain has not been identified. Moreover, several authors have reported no difference for 5HIAA in CSF measured in autistic patients (for an overview see [17]), which is in contradiction to our routine determination of 5HIAA in CSF in patients with autistic disorders where we find a significant percentage of patients with low 5HIAA (unpublished observation). On the other hand, several other laboratories reported recently on isolated low 5HIAA level in CSF from patients with neurological disorders and neuro-psychiatric disturbances, including ASD (without identifying an underlying genetic cause) [25].

Table 2
Polymorphisms and mutations in the *SLC6A4* gene.

Subject	5-HTTLPR	STin2 VNTR	rs6355 (exon 3)
Patient CM	L/L	9/10	Heterozygous c.167G>C (p.Gly56Ala)
Mother	L/S	10/12	Heterozygous c.167G>C (p.Gly56Ala)
Father	L/L	9/10	Wild-type c.167G (p.Gly56)

Mutation designation is based on transcript ID: ENST00000394821 (Ensemble database) with 15 exons. The transcript length is 6335 bps, and the translation length is 672 residues. The numbering starts with 1 at A at the ATG-start codon.

To find a genetic basis that could potentially explain the isolated low serotonin in the CNS (determined indirectly by the lowered 5HIAA concentration in CSF), we performed DNA mutation screening not only for the SERT transporter, encoded by the *SLC6A4* gene, but also for other candidate genes that are directly involved in serotonin metabolism, such as *TPH1*, *TPH2*, and *GCHFR*. In addition to the described genetic variations in *SLC6A4*, there are other gene variants that may have an additional effect or modest influence on autism risk. These include *de novo* copy number variations [26], serotonergic receptor single nucleotide polymorphisms such as in the *HTR3A* gene [27], and also other genetic variants (for a recent overview see [28]). Since the impact of these genetic variations remains unclear or inconclusive, we did not collect these data. Our patient with atypical autism carrying the Gly56Ala/wt genotype in combination with the L/L variant of the *SLC6A4* promoter sequence is thought to have increased expression of both, the normal and mutated allele. We hypothesize that this will predispose to elevated serotonin re-uptake from synaptic clefts, and may be the deciding factor for the autistic phenotype. Furthermore, while the combination of the Ala56 allele with STin2 VNTR remains unclear regarding SERT function, we speculate that the L/S variant, in combination with the Gly56Ala/wt genotype, is the reason why the mother is a non-affected carrier. In addition, we have investigated the *SLC6A4* gene for the Gly56Ala alteration in our current collection of samples from 137 patients with autistic disorder and identified the heterozygous situation in at least five other subjects. Although we do not know at this point the 5HIAA status in CSF for all five patients, at least one has lowered 5HIAA, indicating that low serotonin together with the SERT mutation may be more common in autism spectrum disorder (unpublished observation).

Physiologically, the low CSF 5HIAA levels and diminished urinary 5HIAA excretion in our patient indicate low serotonin turnover. The normalization of urinary 5HIAA excretion after a 5-hydroxytryptophan loading dose in our patient suggested diminished function of the first rate-limiting enzyme of serotonin synthesis, being the peripheral enzyme TPH1 converting tryptophan to 5-hydroxytryptophan. Likewise, the low 5HIAA value in spinal fluid with normalization after treatment by 5-hydroxytryptophan and carbidopa might also suggest low activity by neuronal TPH2. We speculate that an increased serotonin re-uptake by the protein mutant (Gly56Ala) of the serotonin transporters results in an initial overload of serotonin within neuronal tissue and might thus lead to indirect inhibition of serotonin synthesis with consequent reduction of the activity of the rate-limiting enzyme TPH2 (Fig. 3). Further investigation on the mechanisms explaining this indirect inhibition upon neuronal and peripheral TPH1 enzymes warrants subsequent studies. An alternative hypothesis is that the increased serotonin re-uptake initially leads to serotonin accumulation into pre-synaptic cytoplasm with increased transport into vesicles by the vesicular mono-amine transporter (VMAT) and increased degradation by aldehyde dehydrogenase and MAO enzymes. The subsequent enhanced catabolism might in time predispose to overall pre-synaptic serotonin depletion and low serotonin release. In transgenic mice, over-expressing the SERT protein low intraneuronal levels of serotonin and diminished release have been documented [29]. However, more complex adaptive changes mediated by up-regulation of pre-synaptic serotonin auto receptors in the brainstem raphe nuclei (5-HT1A) or at pre-synaptic nerve terminals (5-HT1B/1D) might also provide an explanation for low serotoninergic transmission at these synapses carrying the up-regulated SERT protein [14,30].

In conclusion, our findings in a patient with speech delay and atypical autism associated with gain of function of the *SLC6A4* serotonin transporter gene (Gly56Ala/wt) suggested a final stage of serotonin depletion or reduced serotonin turnover. Moreover, the important clinical and biochemical response following treatment by

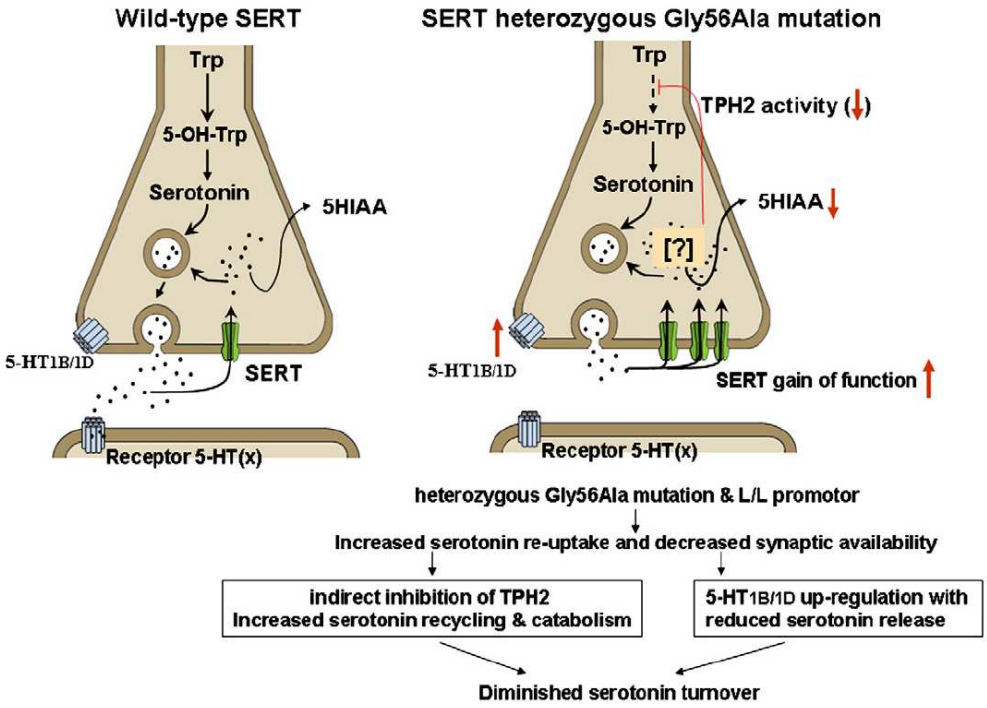

Fig. 3. Possible effects of increased SERT activity. The left side shows normal serotonin synthesis and release followed by serotonin transporter (SERT) mediated re-uptake into the pre-synaptic terminal where either recycling into vesicles or catabolism to 5HIAA by MAO enzymes takes place. On the right, our hypothesis is depicted with low serotonin turnover in the presence of high expression of SERT due to presence of the L/L promoter variant plus gain of function by the SERT 56Ala mutant transporter. The consequent overall increased re-uptake may lead to serotonin accumulation within pre-synaptic terminals (indicated with a question mark). The presence of high intra-neuronal serotonin concentrations is speculated to predispose to secondary diminished serotonin turnover by up to three different mechanisms: (a) indirect inhibition of the rate-limiting serotonin producing enzyme TPH2, (b) increased recycling and degradation of serotonin to 5HIAA resulting in pre-synaptic serotonin depletion, and (c) up-regulation of 5-HT1A autoreceptors within brainstem raphe nuclei (not shown in the figure) and 5-HT1B/1D autoreceptors at pre-synaptic nerve terminals in response to low serotonin concentration within the synaptic space. The up-regulated 5-HT1A and 5-HT1B/1D receptors will diminish axonal excitability and subsequent serotonin release. Abbreviations: SERT: serotonin transporter; Trp: L-Tryptophan; 5-OH-Trp: 5-hydroxytryptophan; TPH2: brain tryptophan hydroxylase 2; 5-HT1A: serotonin 1A autoreceptor in raphe nuclei; 5-HT1B/1D: serotonin autoreceptors in nerve terminals; 5HIAA: 5-hydroxyindoleacetic acid.

5-hydroxytryptophan and carbidopa supports our speculations about one possible pathophysiology of autism.

Supplementary data to this article can be found online at doi:10.1016/j.ymgme.2010.11.162.

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Competing interest: None.

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4.1 Supporting Information on *SLC6A4* gene analysis

Screening for polymorphism in the human SLC6A4 gene

Intronic primers were designed with the online program ExonPrimer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) to flank 15 exons in the human *SLC6A4* gene (listed in Table S1). The PCR was performed in a final volume of 25 µl containing 1 µl of genomic DNA template (100 ng), 1 µl of forward and reverse primer (5 µM), 0.5 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.2 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 1.5 µl 25mM $MgCl_2$, 2.5 µl 10x reaction buffer (Mg^{2+} free from Solis Biodyne) and 5 µl 5x Q-solution (Qiagen). The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95°C for 15 minutes, followed by 35 cycles of denaturation 95°C for 1 minute, annealing 55°C for 45 seconds and extension at 72°C for 1 minute, a final extension was performed at 72°C for 10 minutes and termination at 4°C. The same primers as mentioned above (listed in Table S1) were used to directly sequence the amplified PCR products with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing PCR was performed in a 10 µl reaction mixture consisting of 0.5 µl PCR product, 0.8 µl primer (5 µM), 1.5 µl BigDye and 1.25 µl 5xsequencing buffer (both BigDye and 5xsequencing buffer from BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems)) using standard thermal cycling: (activation at 95°C for 1 min, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 3 minutes and termination at 4°C). After end run 15 µl H_2O was added to the sequencing reactions before purified by gel filtration (using MultiScreen HV 96-well filter plates (Millipore) with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing results were compared with the wild-type sequence of the *SLC6A4* gene using the Mutation Surveyor (Demo) Software v3.20 from SoftGenetics (Transcript ID: ENST00000394821 or accession number NM_001045.4).

Genotyping of SLC6A4 gene-linked polymorphic promoter region (5-HTTLPR)

PCR was used to amplify the polymorphism in the *SLC6A4* gene-linked polymorphic promoter region (5-HTTLPR) consisting of a 44 base pair (bp) insertion or deletion. Two oligonucleotide primers previously described in (Esterling et al., 1998) (forward 5'-CCGCTCTGAATGCCAGCACCTAAC-3' and reverse 5'-AGAGGGACTGAGCTGGAC AACCAC-3') were used to generate two 5-HTTLPR allele-specific fragments: short (S) allele 479 bp and long (L) allele 523 bp. The PCR was performed in a final volume of 25 µl

containing 1 µl of genomic DNA template (100 ng), 1 µl of forward and reverse primer (5 µM), 0.5 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.2 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 1.5 µl 25mM $MgCl_2$, 1.5 µl 10x reactionsbuffer (Mg^{2+} free from Solis Biodyne) and 5 µl 5x Q-solution (Qiagen). The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95°C for 15 minutes, 35 cycles of denaturation 95°C for 30 seconds, annealing 61°C for 30 seconds and extension at 72°C for 1 minute, a final extension at 72°C for 10 minutes and termination at 4°C. The PCR products were run on a 1.5% agarose gel and visualized under ultraviolet (UV) light in the presence of GelRed 10,000X in water (Biotium).

Genotyping of STin2 VNTR

PCR amplification of the VNTR region in intron 2 was performed by using two oligonucleotide primers previously described in (Cook et al., 1997), HTT2X 5'-TGGATTTCCTTCTCTCAGTGATTGG-3' and HTT2Y 5'-TCATGTTCCTAGTCTTACGC CAGTG-3'. The primers amplify fragments at different length according to the number of copies present in intron 2: 345 bp represent 9 copies, 360 bp 10 copies and 390 bp 12 copies. The PCR was performed in a final volume of 25 µl containing 1 µl of genomic DNA template (100 ng), 1 µl of forward and reverse primer (5 µM), 0.5 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.2 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 1.5 µl 25mM $MgCl_2$, 1.5 µl 10x reactionsbuffer (Mg^{2+} free from Solis Biodyne) and 5 µl 5x Q-solution (Qiagen). The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95°C for 15 minutes, 35 cycles of denaturation 95°C for 15 seconds, annealing 57°C for 30 seconds and extension at 72°C for 30 seconds, a final extension at 72°C for 5 minutes and termination at 4°C. The PCR products were run on a 1.5% agarose gel and visualized under ultraviolet (UV) light in the presence of GelRed 10,000X in water (Biotium).

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Table S1.

Primers used for PCR-amplification and sequencing of 5-HTTLPR, STin2VNTR and the 15 exons of the human *SLC6A4* gene

Region	Primer Sequence (forward and reverse)	Product size (bp)
5HTTLPR	5'-CCGCTCTGAATGCCAGCACCTAAC-3'	479 (S) or 523 (L)
	5'-AGAGGGACTGAGCTGGACAACCAC-3'	
HTT2X	5'-TGGATTTCCTTCTCTCAGTGATTGG-3'	345 (9 copies), 360 (10 copies) or 390 (12 copies)
HTT2Y	5'-TCATGTTCCTAGTCTTACGCCAGTG-3'	
Exon 1	5'-GCCTCAAAGTGACGCAAAA-3'	258
	5'-AGGCGGGGAAGAAGGTC-3'	
Exon 2	5'-CTCCCAGTGGAGGCACAG-3'	264
	5'-CCGGCTGTGTCCAGTCTAT-3'	
Exon 3	5'-CATGTAGCAAATAGGATGT -3'	732
	5'-CAGGTCACAGCCCACC -3'	
Exon 4	5'-AGTCACAGATTGGTTGGGC-3'	298
	5'-GATTCCAGAAGAAGGTCC-3'	
Exon 5	5'-GTTTCAGCCTTTTGCCGTT-3'	359
	5'-TTTTAAGAAGCCAAACCCC-3'	
Exon 6	5'-CCTCAAAGGAGCAGCACAG-3'	329
	5'-TGTGTTTGGTGTGGAAGGA-3'	
Exon 7	5'-CTCCCTTCTCCTTCTCCCC-3'	306
	5'-AGCCACATTTCAGTTGTCC-3'	
Exon 8	5'-TCTGCACTTAGCCACATGG-3'	283
	5'-CCAATCACCTTCCTCCACA-3'	
Exon 9	5'-CACGGACAGATGCTCCTTA-3'	317
	5'-CCTTCTTTGGAAAATTTCA-3'	
Exon 10	5'-TGGCTGAGTTTTCCTCTGT-3'	261
	5'-TGAGTCCAGCTGAGTCTTC-3'	
Exon 11	5'-CTTACCCCTCCCTCCTGTT-3'	311
	5'-GGCACTGTGTGAGATGGAA-3'	
Exon 12	5'-CCCCTGCAGGTCTCTGTAG-3'	241
	5'-CAGCCCTTTGCTACTGTGC-3'	
Exon 13	5'-CTCTCCCAGAGAATCAGGG-3'	265
	5'-GCCCAGCCATTACAATTCA-3'	
Exon 14	5'-TGGTGTTTGTGGTATCGGA-3'	358
	5'-CATGCCTCCTGGTGAATCT-3'	
Exon 15	5'-CCAACTCGCTCTTAGATGT-3'	491
	5'-ATCCTAACTGATGATAGGG-3'	

5

Autism spectrum disorder associated with low serotonin in CSF, functional mutations in the *SLC29A* and co-occurrence of mutations in serotonin and/or autism related genes

Running title: *SLC29A* mutations associated with ASDs and low or normal 5-HIAA in CSF

This part is a manuscript in preparation as Adamsen et al.

5 Autism spectrum disorder associated with low serotonin in CSF, functional mutations in the *SLC29A* and co-occurrence of mutations in serotonin and/or autism related genes

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5.1 Abstract

Using a genetic candidate gene approach and whole exome sequencing, we identified in two unrelated individuals with autism spectrum disorder (ASD) and isolated low serotonin in brain as reflected by the serotonin end-metabolite 5-hydroxindolacetic acid (5-HIAA) in CSF - a combination of heterozygous non-synonymous mutations in serotonin-related and/or autism-associated genes. Besides the previously reported codon-alterations from association studies, we newly identified in the plasma membrane monoamine transporter gene *SLC29A4*, encoding a putative serotonin re-uptake transporter protein PMAT, the alterations p.A138T or p.D326E with reduced transporter activity. DNA sequencing of *SLC29A4*/PMAT in 125 individuals with ASD and approximately 300 unaffected control subjects revealed the non-synonymous heterozygous alteration p.M24L or p.D29G in seven additional subjects with ASD (and not in 300 control subjects). According to a hypothetical "genetic accumulation-model" where the affected subjects must differ from non-affected family members (parents and siblings) in the sum of all mutations, the two ASD patients showed additional alterations in mainly serotonin homeostasis, but also other candidate genes that have previously been linked to ASD and/or intellectual disability. Our findings link a combination of mutations in several serotonin-related genes to ASD and mental retardation concomitant to low serotonin in CNS.

5.2 Introduction

Autism spectrum disorders (ASDs), which includes Kanner autism, Asperger's syndrome and pervasive developmental disorder – not otherwise specified (PDD-NOS), are a group of complex, lifelong neurodevelopmental disorders characterized by severe deficits within three behavioural domains: 1) social interaction, 2) verbal and non-verbal communication as well as 3) repetitive, restrictive or stereotypic behavior (Muhle et al., 2004, Geschwind, 2009). Numerous, family and twin studies have reported that ASDs are highly heritable disorders suggesting that approximately 90 % of variance is attributable to genetic factors (Levy et al., 2009). Despite, the strong genetic evidence, the identity and number of genes involved or responsible for ASDs are not yet known (Muhle et al., 2004).

Nevertheless, it is generally assumed that the genes involved in the pathogenesis of autism are implicated in the overall processes of neuronal signal transmission, including molecules such as synaptic scaffolding proteins, cell adhesion molecules, protein involved in second-messenger systems, secreted proteins, receptors and transporters (Castermans et al., 2010) and that this involvement takes place in multiple brain regions including the frontal lobes, anterior temporal lobes, caudate and cerebellum (Abrahams and Geschwind, 2010).

One aspect of ASDs which has been consistent and well-studied over the years is the elevated blood serotonin (hyperserotoninemia) found in one-third of the individuals with autism (Lam et al., 2006). Serotonin plays an important role in the early neural development thus the high levels of serotonin has been suggested to cause loss of serotonin terminals in cerebral cortex via a negative feedback system medicated by serotonin receptors and subsequent influencing the neuronal development (Lam et al., 2006, Whitaker-Azmitia, 2001, Boylan et al., 2007).

Furthermore, studies have shown that patients diagnosed with ASDs demonstrated clinical improvements upon administration of selective serotonin re-uptake inhibitors (SSRIs) (Veenstra-Vanderweele et al., 2009, Buitelaar and Willemsen-Swinkels, 2000).

Altogether, suggestion that an unbalance in peripheral and central serotonin homeostasis is of special interst for ASDs thus supporting the common perception that the serotonergic neurotransmission represents a logical candidate for autism pathology (Bartlett et al., 2005, Anderson et al., 2009). Due to the serotonin hypothesis of autism, the genes which encodes for brain serotonin metabolism and neurotransmission have received particular more attention than other categories of genes (Scott and Deneris, 2005).

In a recent published paper, we demonstrated that isolated low brain serotonin as reflected by the serotonin end-metabolite 5-hydroxindolacetic acid (5-HIAA) in CSF is associated with atypical autism (PDD-NOS) and the functional heterozygous p.G56A mutation in the serotonin re-uptake transporter (SERT) gene *SCL6A4* in combination with a homozygous long (L/L) 5-HTT gene-linked polymorphic promoter (5-HTTLPR) region (Adamsen et al., 2010). Interestingly, daily treatment with serotonin precursor 5-hydroxytryptophan and aromatic amino acid decarboxylase (AADC) inhibitor carbidopa led to clinical improvements and normalization of the 5-HIAA levels in the CSF and urine indicating that the brain serotonin turnover normalized (Ramaekers et al., 2001).

5.3 Results and Discussion

To follow up, this newly discovered association with promising treatment possibilities, we screened genomic DNA from a Caucasian cohort of 125 unrelated patients diagnosed with ASDs and unknown, normal or isolated low 5-HIAA in the CSF by first using a genetic candidate gene approach including genes encoding for SERT, plasma membrane monoamine transporter (PMAT) and GTP cyclohydrolase feedback regulatory protein (GFRP), as they have all been found to be directly involved in serotonin homeostasis. The GFRP/*GCHFR* and PMAT/*SLC29A4* genes were included even though thus far no mutations have been identified within these two genes. Mutations in the genes encoding for aromatic amino acid decarboxylase (AADC) and monoamine oxidase A (MAO-A) were ruled out due to normal catecholamines and homovanillic acid (HVA) levels in the CSF. All exons and exon-boundaries were sequenced from the following genes: SERT (*SLC6A4*, OMIN: 182138, ENST00000394821), PMAT (*SLC29A4*, OMIM: 609149, ENST00000297195) and GFRP (*GCHFR*, OMIM: 602437, ENST00000260447) (**Supplementary Information**).

In 7.2 % of the 125 ASDs affected patients with unknown, normal or isolated low 5-HIAA in the CSF, we identified four non-synonomous mutations within the *SLC29A4* gene encoding for PMAT: p.M24L, p.D29G, p.A138T and p.D326E, respectively (**Figure 23**). The individual exon-sequening turned out to be negative for mutations within the *GCHFR* gene encoding GFRP. However, 4 % of the 125 ASDs affected patients with unknown, normal or isolated low 5-HIAA in the CSF were carrying the heterozygous p.G56A alteration in the *SLC6A4* gene encoding for SERT, which previously has been associated with autism and rigid-compulsive behaviours (Sutcliffe et al., 2005). In one patient, we found both a mutation within the SERT/*SLC6A4* gene (p.G56A) and the PMAT/*SLC29A4* gene (p.D326E).

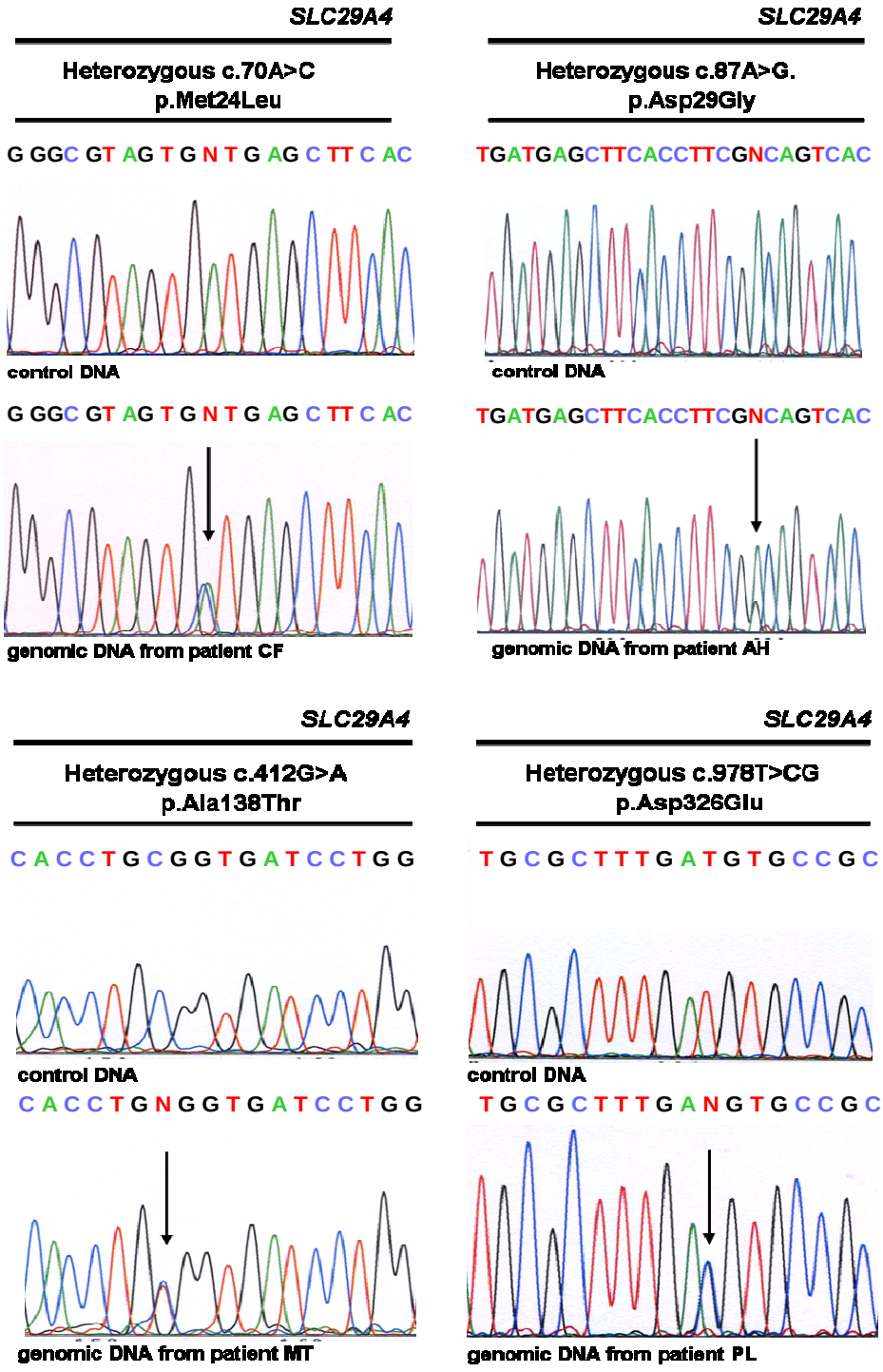

Figure 23: Four newly identified PMAT mutations in the *SLC29A4* gene

Out of 125 patients diagnosed with ASDs and normal or isolated low 5-HIAA in the CSF, 9 patients were carrying a heterozygous mutation in the *SLC29A4* gene encoding for PMAT: p.M24L, p.D29G, p.A138T and p.D326E, respectively. p.M24L was found in few patients with autism (two with normal 5-HIAA), p.D29G was found in two patients with ASDs (one with normal 5-HIAA), p.A138T was found in patient MT with ASD and isolated low 5-HIAA and finally, p.D326E was found in patient PL with ASD and isolated low 5-HIAA. Moreover, PL was found to carry the heterozygous p.G56A mutation in the *SLC6A4* gene encoding for SERT.

Whereas, the SERT-G56A alteration is a known mutation with gain of function regarding serotonin transport (Sutcliffe et al., 2005), mutations within the PMAT/*SLC29A4* gene have not prior been described thus making the four newly identified PMAT-mutations first of their kind. Several studies have suggested strong evidence for linkage between autism and a region on chromosome 7q22-7q32 (Abrahams and Geschwind, 2008) which is interesting in terms of our findings as the *SLC29A4* gene is located on chromosome 7p22.1 (Engel et al., 2004).

Using genomic DNA from an independent group of approximately 300 unaffected control subjects, we immediately ruled out the possibility that these four newly identified PMAT-mutations were single nucleotide polymoprhisms (SNPs) (**Supplementary Information**).

Apart from PMAT-A138T and PMAT-D326E, the serotonin re-uptake transporter mutations within the PMAT/*SLC29A4* gene were all found in more than one ASD affected patient (**Table 1**). PMAT-M24L and PMAT-D29G, which are both located in the N-terminal tail (**Figure 24**), were found in five and two ASDs affected patients, respectively with normal or unknown 5-HIAA levels in the CSF (**Table 1**).

Table 1: Serotonin re-uptake transporter mutations found in 125 ASDs patients diagnosed with normal, unknown or isolated low 5-HIAA in the CSF

Gene	Protein	Heterozygous mutation	Patient (ASDs)	5-HIAA in CSF (nmol/L)
SLC6A4	SERT	p.G56A	CM (PDD-NOS)	Low (Adamsen et al., 2010)
SLC6A4	SERT	p.G56A	PL (PDD-NOS) [a]	Low (Table 2)
SLC6A4	SERT	p.G56A	VE (Asperger's)	Normal but in low range
SLC6A4	SERT	p.G56A	ARN (PDD-NOS)	Unknown
SLC6A4	SERT	p.G56A	CAT (Autism)	Unknown
SLC29A4	PMAT	p.M24L	CF (Autism)	Normal
SLC29A4	PMAT	p.M24L	PM (Autism)	Normal
SLC29A4	PMAT	p.M24L	KAP (Autism)	Unknown
SLC29A4	PMAT	p.M24L	FER (Autism)	Unknown
SLC29A4	PMAT	p.M24L	7542 (Autism)	Unknown
SLC29A4	PMAT	p.D29G	AH (PDD-NOS)	Normal
SLC29A4	PMAT	p.D29G	ACI (Autism)	Normal
SLC29A4	PMAT	p.A138T	MT (Asperger's)	Low (Table 2)
SLC29A4	PMAT	p.D326E	PL (PDD-NOS) [a]	Low (Table 2)

[a] Patient PL was found both to carry a heterozygous mutation within the SERT/*SLC6A4* gene (SERT-G56A) and the PMAT/*SLC29A4* gene (PMAT-D326E), respectively.

In contrast, PMAT-A138T which is located in the third membrane-spanning domain (**Figure 24**), was found in patient MT diagnosed with Asperger's syndrome and isolated low 5-HIAA in the CSF (**Table 2**). Likewise, PMAT-D326E which is located in the third cytoplasmic loop (**Figure 24**), was found in patient PL diagnosed with PDD-NOS and isolated low 5-HIAA in the CSF (**Table 2**). In addition, patient PL was found to carry the functional heterozygous SERT-G56A alteration (not shown).

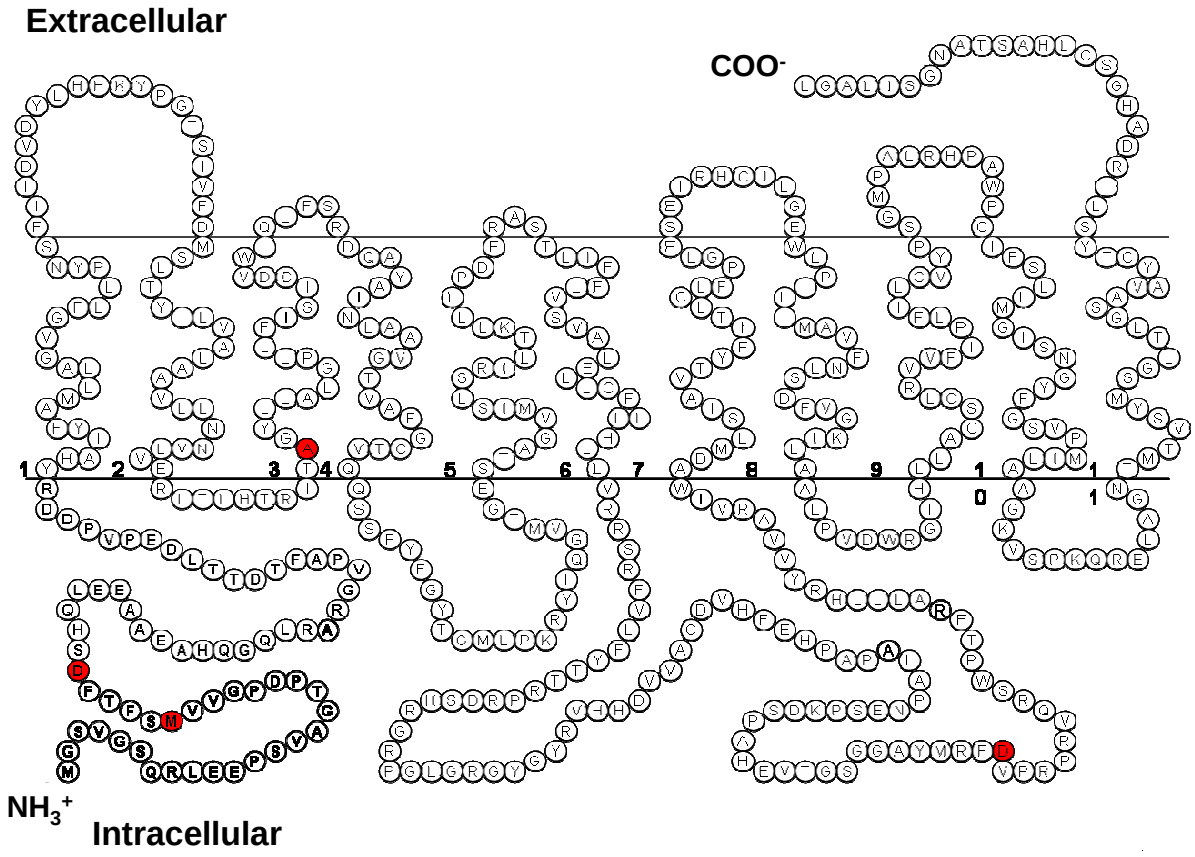

Figure 24: Position of four newly identified alternations in the secondary structure of PMAT

The *SLC29A4* gene encoding the serotonin re-uptake transporter PMAT, is translated into a plasma membrane transporter protein composed of 530 amino acids. Hydropathy analysis has predicted the presence of 11 membrane-spanning domains, a cytoplasmic N-terminus (NH_3^+) and an extracellular C-terminus (COO^-). The four newly identified mutations in the PMAT gene *SLC29A4* (PMAT-M24L, PMAT-D29G, PMAT-A138T and PMAT-D326E) are marked by filled red circles. Two of the PMAT mutations (PMAT-M24L and PMAT-D29G) are located in the N-terminal tail, whereas one is located in the third membrane-spanning domain (PMAT-A138T) and one in the third cytoplasmic loop (PMAT-D326E), respectively. The four PMAT mutations which were all tested to be negative in approximately 300 independent and unaffected control subjects were found in a total of 9 patients diagnosed with ASDs and normal (PMAT-M24L and PMAT-D29G) 5-HIAA in CSF or isolated low 5-HIAA in CSF (PMAT-A138T and PMAT-D326E).

To investigate in further details, the two PMAT-mutations: A138T and D326E, respectively which were associated with ASDs and isolated low 5-HIAA in the CSF, we analyzed the PMAT protein quantification, subcellular localization in MDCK (Madin-Darby canine kidney) cells and radiolabeled substrate transport activities of the two mutated PMAT proteins and compared them with wild-type PMAT (**Supplementary Information**) (**Figure 25**).

The subsequently, cell surface biotinylation and Western blot analysis demonstrated comparable plasma membrane expression of PMAT-wild-type (wt), PMAT-A138T and PMAT-D326E protein (**Figure 25A**).

When expressed in MDCK cells and observed by confocal microscopy, the two mutated PMAT proteins were located in the plasma membrane similar to wild-type PMAT (wt) (**Figure 25B**). Demonstrating that these two PMAT mutations do not influences the localization of the serotonin re-uptake transporter protein in the plasma membrane.

To ascertain the functional properties of the two PMAT mutations, A138T and D326E, respectively, we screened a variety of radiolabeled substrates including serotonin, dopamine and MPP^+ (1-methyl-4-phenylpyridinium[1]) for uptake activity (% of wild-type) **(Figure 25C**). The functional studies showed that both PMAT-A138T and PMAT-D326E exhibit reduced transport activity towards serotonin, dopamine and MPP^+ compared to wild-type PMAT (wt) (**Figure 25C**).

Despite, the reduced transport activity compared to wild-type PMAT, the two PMAT mutant proteins exhibit similar transport efficiencies for serotonin, dopamine and MPP^+ thus demonstrating similar substrate specificity and thereby not functionally distinct from each other (**Figure 25C**). Consequently, in terms of the substrate uptake activity, the localization of the two PMAT mutations in the third membrane-spanning domain and cytoplasmic loop, respectively appeared to be irrelevant.

Whereas SERT-G56A is known to cause an up-regulation regarding serotonin transport (gain of function), our functional data suggested that the two PMAT mutations (PMAT-A138T and PMAT-D326E) are not “loss of function” but rather showed a reduced transport activity towards serotonin (**Figure 25C**).

[1] MPP^+ (1-methyl-4-phenylpyridinium) is a neurotoxin that causes Parkinson’s disease by selectively damaging dopaminergic nerve terminals (Engel et al., 2004).

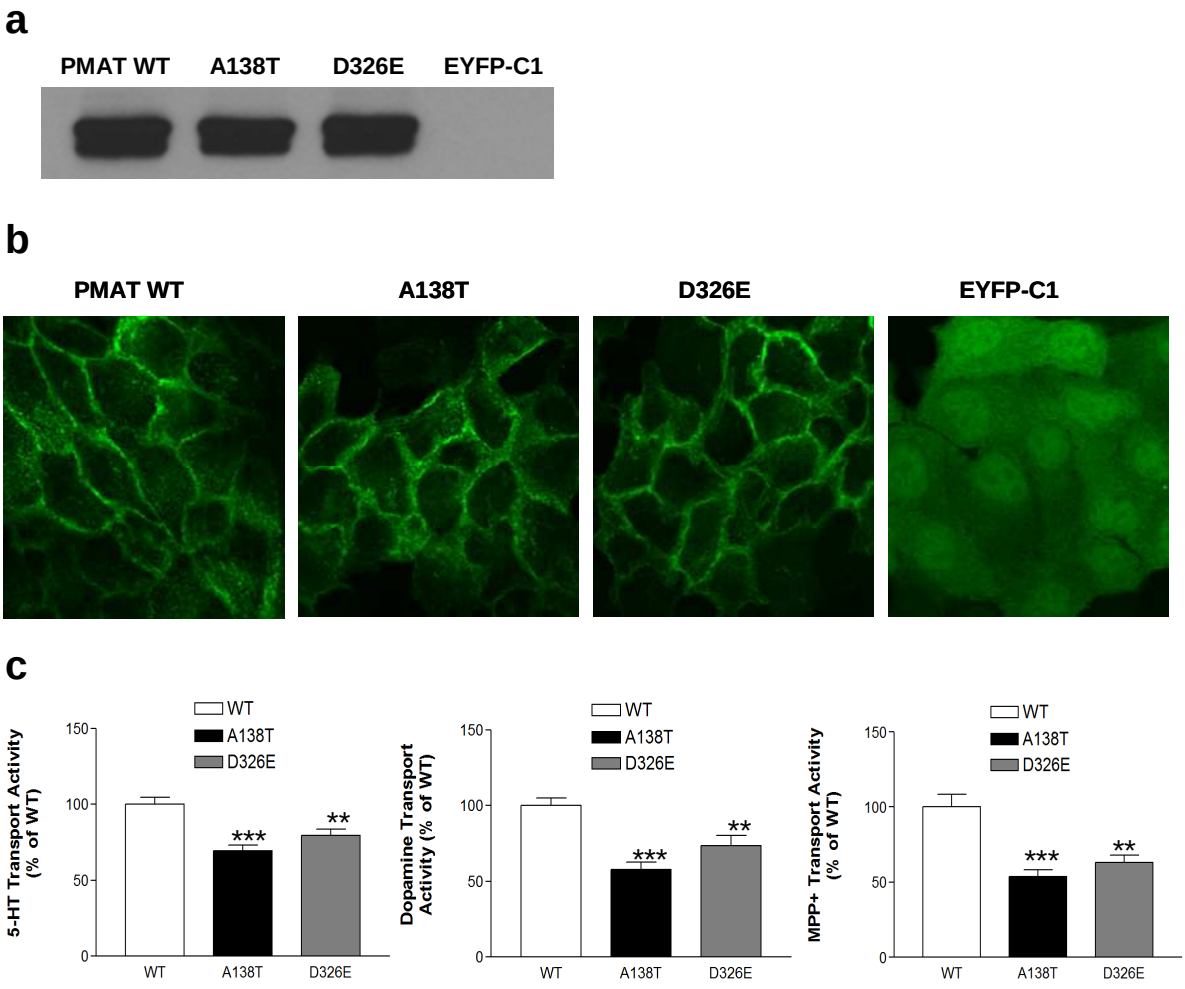

Figure 25: PMAT protein expression, membrane localization and substrate transport activity

A: Cell surface biotinylation and Western blot analysis demonstrated comparable plasma membrane expression of PMAT-wild-type (wt), PMAT-A138T and PMAT-D326E protein. Empty pEYFP-C1 vector was transfected into MDCK cells to obtain the control cell line. Double or multiple protein bands around the expected molecular size (~75 kDa) were observed for the YFP tagged PMAT proteins, which could be due to differential glycosylation of PMAT. B: Confocal microscopy imaging of MDCK cells expressing PMAT-wild-type, PMAT-A138T, PMAT-D326E and empty pEYFP-C1 vector. The two mutated PMAT proteins (A138T and D326E) were shown to be located in the plasma membrane similar to wild-type PMAT (wt). C: The serotonin (5-HT), dopamine and MPP^+ transport activity were significantly reduced in the PMAT-A138T and PMAT-D326E transfected MDCK cells, respectively compared to the MDCK cells transfected with PMAT-wild-type. PMAT-wild-type (white bar), PMAT-A138T (black bar) and PMAT-D326E (gray bar). For all the transport activity experiments, the values are indicated in mean ± S.D. from three independent experiments (n=3) with different cell passages. For each experiment, uptake was carried out in triplicates in three different wells on the same plate. Significant difference from the corresponding value for PMAT-wild-type (100 % transport activity) is indicated by asterisks: **, $p < 0.01$ and ***, $p < 0.001$ (Student's t test).

Both the SERT and PMAT gene variants, we have identified thus far have all been present in their heterozygous state (Figure 23). From genomic DNA sequencing analysis of the parents of the affected ASDs patients (PMAT-A138T and PMAT-D326E/SERT-G56A), we know that the SERT and PMAT mutations are inherited from unaffected patents and that "simple" haplotype association/analysis can not explain heritability of these disorders (not shown).

Hence, to understand the underlying genetic linking ASDs with isolated low 5-HIAA in the CSF with serotonin re-uptake transporter mutations, we performed whole exome sequencing on the two ASDs affected patients carrying PMAT-A138T and PMAT-D326E (and SERT-G56A), respectively plus their unaffected parents and sibling (**Supplementary Information**).

By including the unaffected parents and sibling (in both cases a sister) we particular aimed to address the question why the parents from whom the affected patient has inherited the heterozygous serotonin re-uptake transporter mutation (or mutations) is unaffected.

To analyse the whole exome sequencing data we focused on candidate genes which have been published to be involved in serotonin and/or autism (for more information about the search strategy and selection criteria see **Supplementary Information**).

The candidate gene search led to two lists: one containing the candidate genes published to be involved in serotonin (**Supplementary Table S2**) and one with the candidate genes published to be involved in autism, ASDs and ASD-related syndromes (**Supplementary Table S3**).

We filtered the whole exome sequencing data for non-synonymous amino acid substitutions within serotonin-related and/or autism-associated candidate genes (**Supplementary Table S2 and Supplementary Table S3**). Furthermore, we excluded using NCBI population diversity, the non-synonymous mutations with an allele frequency higher than 1.

The result of the whole exome sequence analysis and thereby the sum of all the non-synonymous amino acid substitutions in family MT and PL with an allele frequency <1 are shown in **Table 3**.

Table 3: Non-synonymous findings in patient MT and PL plus their parents and sibling within candidate genes involved in serotonin and/or ASDs

Gene name	RefSeq IDs	Codon exchange	Patient MT	Mother	Father	Sibling	Associated disease	Allele frequency (<1)
SLC29A4	Unknown	p. A138T	Het	Het	Wt	Wt	Serotonin	-
SLC6A4	rs6355	p.G56A	Wt	Wt	Wt	Wt	Serotonin	C/G 0.050
5-HTTLPR	n.d.	-	L/S	L/L	L/S	n.m.	Serotonin	-
STin2VNTR	n.d.	-	12/12	12/12	10/12	n.m.	Serotonin	-
ITGB3	rs5918	p. L59P	Het	Wt	Het	Wt	ASD	C/T 0.239
CNTNAP2	Unknown	p. N407S	Het	Het	Wt	Wt	ASDs/ASD-related syndromes	-
HTR3D	rs112096436	p.G329S[a]	Het	Wt	Het	Het	Serotonin	-
CYFIP1	rs7170637	p.G389S[b]	Het	Wt	Homo	Het	Autism	A/G 0.267
DISC1	rs821616	p. S704C	Het	Het	Homo	Homo	ASDs	A/T 0.383
CACNA1C	rs10774053	p. M1821V[c]	Het	Homo	Wt	Het	ASDs/ASD-related syndromes	A/G 0.435
CACNAIC	rs10848683	p. P1820L	Het	Homo	Wt	Het	ASDs/ASD-related syndromes	C/T 0.319
HTR3B	rs1176744	p. Y129S	Het	Het	Wt	Het	Serotonin	G/G 0.088
CFTR	rs213950	p.V470M[d]	Homo	Homo	Homo	Homo	Autism	A/A 0.208
Gene name	**RefSeq IDs**	**Codon exchange**	**Patient PL**	**Mother**	**Father**	**Sibling**	**Associated disease**	**Allele frequency (<1)**
SLC29A4	Unknown	p.D326E	Het	Wt	Het	Wt	Serotonin	-
SLC6A4	rs6355	p.G56A	Het	Wt	Het	Wt	Serotonin	C/G 0.050
5-HTTLPR	n.d.	-	L/S	S/S	L/L	n.m.	Serotonin	-
STin2VNTR	n.d.	-	10/12	12/12	10/10	n.m.	Serotonin	-
SLC22A1	rs628031	p. M408V	Homo	Homo	Het	Homo	Serotonin	G/G 0.417
HTR3D	rs1000952	p. R177H[e]	Homo	Het	Het	Het	Serotonin	T/T 0.438
CYFIP1	rs7170637	p. G389S[b]	Het	Wt	Het	Wt	Autism	A/G 0.267
DISC1	rs821616	p. S704C	Het	Wt	Het	Wt	ASDs	A/T 0.383
HTR3D	rs6443930	p. G36A[f]	Het	Wt	Het	Het	Serotonin	-
CACNA1C	rs10848683	p. P1820L	Het	Het	Homo	Homo	ASDs/ASD-related syndromes	C/T 0.319
DISC1	rs3738401	p. R264Q	Het	Wt	Homo	Het	ASDs	A/G 0.416
SLC22A1	rs683369	p.L160F[g]	Homo	Homo	Homo	Homo	Serotonin	C/C 0.345
CACNA1C	rs10774053	p. M1821V[c]	Homo	Homo	Homo	Homo	ASDs/ASD-related syndromes	G/G 0.565
CFTR	Rs213950	p.V470M[e]	Het	Het	Het	Wt	Autism	A/G 0.458

[a] NP_001138615.1, [b] NP_001028200.1, [C] NP_001161096.1, [d] NP_000483.3, [e] NP_001138615.1, [f] NP_872343.2, [g] NP_003048.1. n.d.: Not defined, n.m.: Not measured.

In parallel, we measured the serotonin levels both in blood (ng/ml) and platelets (ng/10^9) in the two ASDs affected patients MT and PL (**Table 2**) plus detected common non-coding VNTR (5-HTTLPR and STin2 VNTR) polymorphisms within the *SLC6A4* gene (**Table 3**).

Table 2: Summary of metabolic findings in patient MT (Asperger's) and PL (PDD-NOS)

Patient	Age (yrs)	5-HIAA (nmol/l)	HVA (nmol/l)	HVA/5-HIAA (ratio)	Serotonin	
					Blood (ng/ml)	Platelets (ng/10^9)
MT	9.6	64.7	389.6	6.0	287	1041
PL	6.2	73.6	399.8	5.4	221	675
Ref *	5-10	133 (88-178)	523 (144-801)	1.5-3.5	50-220**	125-500**

* Reference values and ranges see also N. Blau, L. Bonafe, M.E. Blaskovics, Disorders of phenylalanine and tetrahydrobiopterin metabolism Physician's Guide to the Laboratory Diagnosis of Metabolic Diseases Springer 2nd Edition, Blau, N., Duran, M., Blaskovics, M.E., Gibson, K.M. (eds) (2003) 89-106.

** Reference values see Burtis, C.A., Ashwood, E.R., Tietz Fundamentals of Clinical Chemistry, 5th edition, 2001.

As shown in **Table 2** both patient MT and PL exhibit low isolated 5-HIAA levels in the CSF and elevated serotonin levels in the blood as well as in the platelets compared to the reference values. The elevated serotonin levels in the blood and platelets support the previous studies showing that one-third of all ASDs affected individuals have hyperserotoninemia (Cross et al., 2008, Leboyer et al., 1999). Moreover, the PCR-analysis of the non-coding VNTR polymorphisms within the *SLC6A4* gene shown in **Table 3** demonstrated that patient MT and PL are both carrying the L/S variant of the *SLC6A4* promoter region in combination with 12/12 STin2 VNTR and 10/12 STin2 VNTR, respectively.

Using a hypothetical "genetic accumulation-model" (**Figure 26**), where all the non-synonymous findings from the serotonin-related and/or autism-associated candidate gene lists (**Supplementary Table S2 and Supplementary Table S3**) with an allele frequency <1 were amalgamated ("total gene hits") in the affected patients (MT and PL) plus their unaffected family members (parents and sisters), then the cumulative genetic burden of the two ASD affected patients showed more "serotonin gene hits" indicating alterations in mainly serotonin homeostasis compared to the other family members (**Table 3 and Figure 26**).

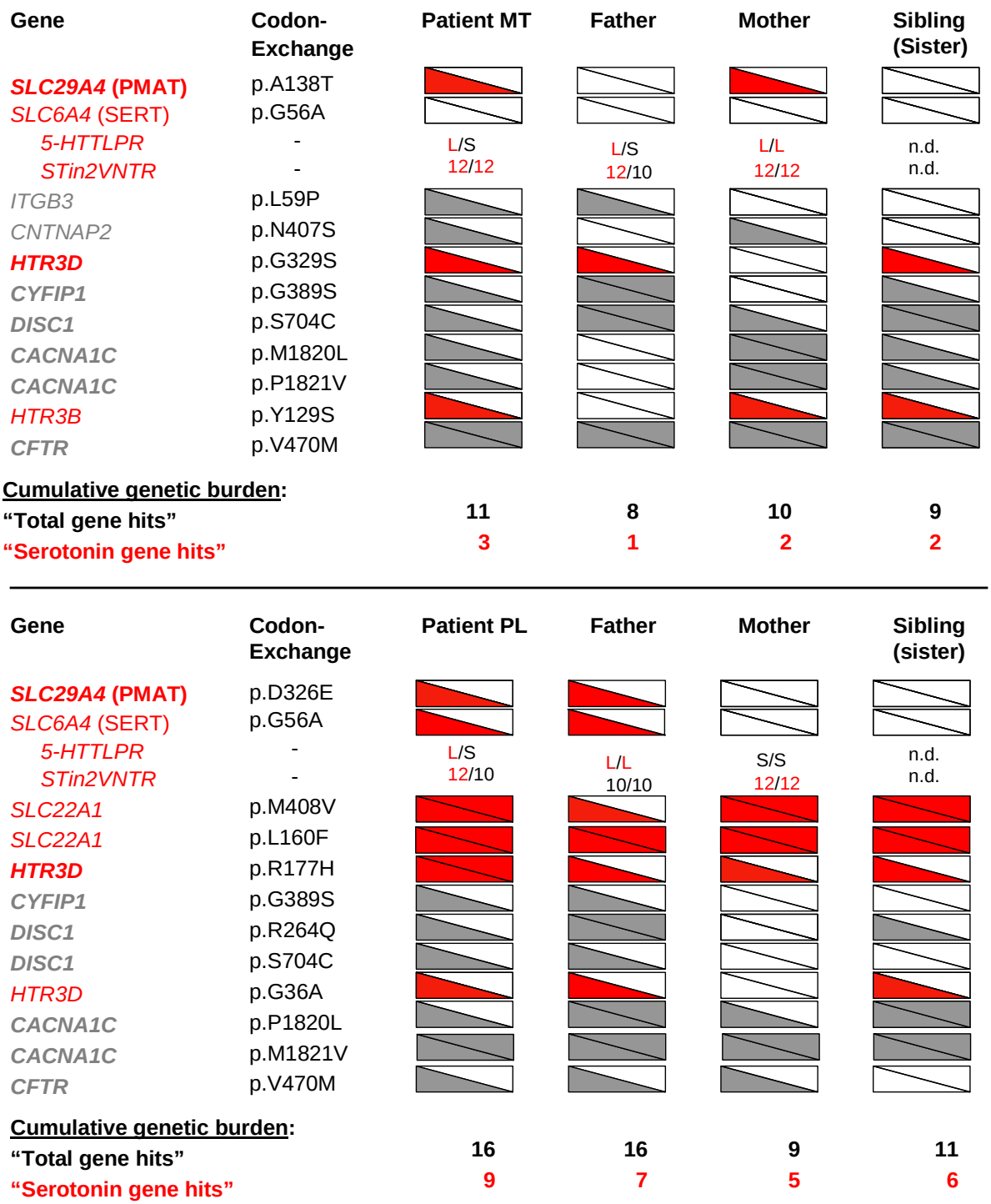

Figure 26: Cumulative genetic burden using a "genetic accumulation-model"

When all the non-synonymous findings from the serotonin-related (in red) and autism-associated (in gray) candidate gene lists with an allele frequency <1 are weighed, the cumulative genetic burden shown more "serotonin gene hits" in the two index patients compared to their healthy family members. The two VNTR polymorphisms (5-HTTLPR and STin2VNTR) in the *SLC6A4* gene are not included in the cumulative genetic burden as their functions have been found to be complex and controversial. In bold are the genes, which are, mutated in both patient MT and PL. Full-triangle: homozygous, half-triangle: heterozygous and empty-triangle: wild-type. n.d. not determined.

Most interestingly, the whole exome sequence analysis revealed that patient MT among others was carrying heterozygous mutations in the autism-associated *CNTNAP2* gene encoding for contactin-associated protein-like 2 and *ITGB3* gene encoding for integrin β-3, respectively (**Table 3**). Whereas, the heterozygous variant in the *CNTNAP2* gene (p.N407S) was not detected in the NCBI dsSNP database and negative in the 300 unaffected control subjects (**Supplementary Information**), the heterozygous variant in the *ITGB3* gene (p.L59P) was a known variant which previously has been published to be associated with austism in co-occurrence with the *SLC6A4* gene primarily in males (Weiss et al., 2006a, Weiss et al., 2006b, Ma et al., 2010, Napolioni et al., 2011, Coutinho et al., 2007).

Despite, a tendency towards a genetic accumulation of mutations within serotonin re-uptake transporter genes in co-occurrence with mutations in autism-associated genes, our whole exome sequencing results, does not explain why the parents from whom the affected patients have inherited the heterozygous serotonin re-uptake transporter mutation (or mutations) is unaffected thus further investigations are necessary in order to elucidate this issue.

Currently, we are increasing the ASDs affected cohort by including more autistic patients and by *in silico* analysis e.g. 1000 Genomes Project. Moreover, we are searching the whole exome sequencing data for *de novo* mutations within patient MT and PL.

Together, our data support the common perception that ASDs are caused by the concurrent effect of several different genes (polygenic mechanism) rather than caused by only one single gene (monogenic cause) (Castermans et al., 2004). Although, environmental factors have not been shown to be a cause of ASDs, they may well act as second “hits” in genetically susceptible individuals (Castermans et al., 2004).

Treatment of one PDD-NOS affected patient with serotonin re-uptake transporter mutation (SERT-G56A) and isolated low 5-HIAA in the CSF has prior been shown to cause an improvement of the clinical features and normalization of the brain serotonin turnover (Ramaekers et al., 2001). In this perspective, it could be interesting to investigate whether all ASDs affected patients with serotonin re-uptake transporter mutations in combination with low brain serotonin would benefit from 5-hydroxytryptophan and carbidopa treatment.

If serotonin abnormalities due to hyperserotoninemia, isolated low 5-HIAA in the CSF or functional mutations in serotonin related genes, could be demonstrated to be essential in the etiology of ASDs, the possibility for new and improved therapies could easier be identified.

5.4 Supplementary Information

Screening for mutations in the human SLC29A4 gene

Intronic primers were designed with the online program ExonPrimer (http://ihg2.helmholtz-muenchen.de/ihg/ExonPrimer.html) to flank the exons in the human *SLC29A4* gene (see Table S1). PCR reactions were performed in a final volume of 25 µl containing 1 µl of genomic DNA template (100 ng), 1 µl of each primer (forward and reverse) (5 µM), 0.5 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.2 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 1.5 µl 25mM $MgCl_2$, 2.5 µl 10x reaction buffer (Mg^{2+} free from Solis Biodyne) and 5 µl 5x Q-solution (Qiagen).

Table S1:

Primers used for PCR-amplification and sequencing of the human *SLC29A4* gene

Region	Primer Sequence (forward and reverse)	Product size (bp)
Exon 2	5'-ATGGATCCCTCAGCCTTCT-3'	285
	5'-ACAGCCCAGCCTGCTTT-3'	
Exon 3	5'-GGTGACTGTAGCCATGCGT-3'	242
	5'-CAGGCCATGATGGAGGAT-3'	
Exon 4	5'-CAGGAGTCAGTGCAGGGG-3'	253
	5'-GGAAACTGGGTGAGTGGGT-3'	
Exon 5	5'-GACTCTGCAGGAGGGGC-3'	263
	5'-GATGGGGTCGGAAGTGG-3'	
Exon 6	5'-TCTCCCAAGTTGACAGGTG-3'	198
	5'-AAGAAGCTTCCAGAGCCCA-3'	
Exon 7	5'-GTCTGTGTGTGGACGGGG-3'	403
	5'-GGGGACAGAGAAGGGGAC-3'	
Exon 8-9	5'-CCCGTGTCTCCTGTCCTC-3'	563
	5'-CAGGGCCTCCCTTGTCAC-3'	
Exon 10	5'-GGATGTGGCTAGAGGCTGT-3'	403
	5'-GTCTAGCCTGGGTTTCCTC-3'	
Exon 11	5'-GTCACCGCACCTCACACC-3'	312
	5'-CACTGAGGTGGGGACAGG-3'	

The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95°C for 15 minutes, followed by 35 cycles of denaturation 95°C for 1 minute, annealing 55°C for 45 seconds and extension at 72°C for 1 minute, a final extension was performed at 72°C for 10 minutes and termination at 4°C. The same forward and reverse primers as mentioned above were used to sequence the amplified PCR products with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

Sequencing PCR was performed in a 10 µl reaction mixture consisting of 0.5 µl PCR product, 0.8 µl primer (5 µM), 1.5 µl BigDye and 1.25 µl 5x sequencing buffer (BigDye and 5xsequencing buffer were both from BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems)) using standard thermal cycling: (activation at 95°C for 1 min, followed by 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, 60°C for 3 minutes and termination at 4°C). After end run 15 µl H_2O was added to the sequencing reactions before purified by gel filtration (using MultiScreen HV 96-well filter plates (Millipore) with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing results were compared with wild-type sequence of the *SLC29A4* gene by using Mutation Surveyor (Demo) Software v3.20 from SoftGenetics (Transcript ID: ENST00000297195 or accession number NM_001040661).

Screening for mutations in the human SLC6A4 gene

Screening of genomic DNA for mutations in the human *SLC6A4* gene as well as detection of non-coding VNTR polymorphisms (5-HTTLPR and STin2 VNTR) in the human *SLC6A4* gene were performed as previously described in (Adamsen et al., 2010).

Screening of 300 control subjects for PMAT-M24L, D29G, A138T and D326E

300 control subjects (genomic DNA kindly provided by PD Dr. Martin Hersberger and Jacqueline Marti-Jaun) were screened for the four newly discovered variants M24L, D29G, A138T and D326E within the *SLC29A4* gene encoding for PMAT to test whether these variants were "simple" single nucleotide polymorphisms (SNPs) or mutations (same primers and PCR conditions as descrived under "**Screening for mutations in the human *SLC29A4* gene**"). The 300 control subjects (600 chromosomes in total giving a polymorphism frequency >0.01 and a <5 % possibility of missing a polymorphism (Collins and Schwartz, 2002)) represented all ages, both sex and with a Caucasian background.

Construction of PMAT Mutations by Site-Directed Mutagenesis

To facilitate the determination of membrane localization, PMAT mutants were constructed using yellow fluorescence protein (YFP)aaA-tagged wild-type human PMAT as template. YFP was tagged at the N-termini of the wild-type and mutant PMAT transporters, as previous studies have shown that the YFP tagging had no effect on substrate selectivity and kinetic behaviors of the transporter (Zhou et al., 2007). The wild-type human PMAT was previously sub-cloned into the YFP vector pEYFP-C1 (Clontech, Palo Alto, CA) (Engel et al., 2004). PMAT-A138T and D326E mutations were introduced into wild-type PMAT/pEYFP-C1 vector by site-directed mutagenesis (PMAT/pEYEP-C1 vector was kindly provided by Dr. Joanne Wang from University of Washington, Seattle).

Primers were designed to introduce threonine at position 138 and glutamic acid at position 326, respectively. A138T_F: 5'-GCACACCAGGATCACCACAGGCTACCTCTTAGC-3' (forward) and A138R_R: 5'-GCTAAGAGGTAGCCTGTGGTGATCCTGGTGTGC-3' (reverse) as well as D326E_F: 5'-CCTACATGCGCTTTGAGGTGCCGCGGCCAAGGG-3' (forward) and D326E_R: 5'-CCCTTGGCCGCGGCACCTCAAAGCGCATGTAG G-3' (reverse). 10 ng of the DNA template (PMAT/pEYFP-C1 vector) was mixed with 1 µl 20 mM deoxyribonucleoside triphosphates (dNTPs), 5 µl 10xreaction buffer G (Solis BioDyne), 1 µl of each primer (forward and reverse) (10 uM) and 1 µl HotGyroPol (Solis BioDyne) in a final volume of 50 ul. The PCR conditions included an initial denaturation cycle (95^{o}C for 15 minute); 15 cycles of denaturation (95^{o}C for 30 seconds), annealing (55^{o}C for 1 minute), and extension (72^{o}C for 6 minutes); a termination at 4^{o}C. PCR products were digested with 10U of DpnI at 37^{o}C for 1 hour to remove the parental DNA followed by transformation into DH5α supercompetent Escherichia coli cells by heat shock (30 minutes on ice, 42^{o}C for 90 seconds, 5 minutes on ice) followed by incubation for 1 hour at 37^{o}C in 500 µl LB-medium. 300 µl of the incubation-mixture was plate on kanamycin/neomycin/agar-plates overnight at 37^{o}C. Positive colonies were confirmed by DNA sequencing using one of the following primers (For A138T with forward: A138T_1F: 5'-CAGGAGTCAGTGCAGGG G-3' and/or reverse: A138T_1R: 5'-GGAAACTGGGTGAGTGGGT-3' and for D326E with forward D326E_1F: 5'-ACAGCCACCGGGACAGGCCA-3' and/or reverse D326E_1R: 5'-ACTCGA GGCCGGGGAACAGG-3') and BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing reactions (0.5 µl DNA, 1.5 µl BigDye and 1.25 µl 5x sequencing buffer both from Terminator v1.1 Cycle Sequencing Kit) were purified by gel filtration (using MultiScreen HV 96-well filter plates (Millipore) with Sephadex G-50 (GE Healthcare)) and

analyzed on a 3130xl Genetic Analyzer (Applied Biosystems). Sequencing results were compared with wild-type sequence of the *SLC29A4* gene by using Mutation Surveyor (Demo) Software v3.20 from SoftGenetics (Transcript ID: ENST00000297195 or accession number NM_001040661).

Stable Expression in MDCK (Madin-Darby canine kidney) Cells

YFP-tagged mutant constructs were transfected into MDCK cells using Lipofectamine 2000 transfection reagent (Invitrogen, Carlsbad, CA). Stably transfected cell lines were obtained by culturing cells in minimal essential medium containing 10% fetal bovine serum and G418 (1000 μg/ml). Empty pEYFP-C1 vector was transfected into MDCK cells to obtain the control cell line. After 2-3 weeks of drug selection, fluorescence-positive cells were purified by a FACS Vantage SE sorter (BD Biosciences, San Jose, CA) at the Cell Analysis Center at the University of Washington, Health Sciences Center. The sorted cells were cultured and maintained in minimal essential medium containing G418 (200 μg/ml).

Confocal Fluorescence Microscopy

To determine the cellular localization of YFP-tagged mutant transporters, ~$2x10^5$ cells were grown on top of microscope cover glass in 6-well plates (Falcon) for 2-3 days until confluent. Cells were mounted onto microscope glass slides with Fluoromount-G (Electron Microscopy Sciences, Hatfield, PA) and visualized with a Leica SP1 confocal microscope equipped with an argon laser as the light source at the Keck Microscopy Facility at the University of Washington. Images were captured by excitation at 488 nm and emission at 515 nm.

Functional Characterization in MDCK cells

Stably transfected MDCK cells were plated in 24-well plates and allowed to grow for 2-3 days until confluent. Growth medium was aspirated and each well was rinsed once with Krebs-Ringer-Henseleit buffer (5.6 mM glucose, 125 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 25 mM HEPES, pH 7.4) and preincubated in the same buffer for 15 min at 37°C. Transport assays were performed at 37°C by incubating cells in KRH buffer containing a ^{3}H-labeled ligand. [^{3}H]MPP^+ (85 Ci/mmol) was obtained from American Radiolabeled Chemicals, Inc. (St. Louis, MO). [^{3}H]5-HT (5-hydroxy-[1,2-^{3}H]tryptamine creatinine sulfate, 28.1 Ci/mmol) and [^{3}H] dopamine (3,4-dihydroxy-[2,5,6-^{3}H]phenylethylamine, 51.3 Ci/mmol) were from PerkinElmer Life Sciences, Inc. All other chemicals were obtained from Sigma (St. Louis, MO).

Uptake was terminated by washing the cells three times with ice-cold KRH buffer. Cells were then solubilized with 0.5 ml of 1N NaOH and neutralized with 0.5 ml of 1N HCl. Radioactivity in the cell lysate was quantified by liquid scintillation counting. Protein concentration in each well was measured using BCA protein assay kit (Pierce) and the uptake in each well was normalized to its protein content. In all studies, cells transfected with an empty vector were served as a background control. Transporter-specific uptake was calculated by subtracting the background uptake in vector-transfected cells.

Isolation of Plasma Membrane Proteins by Cell Surface Biotinylation

Stably transfected MDCK cells were plated onto 60 mm plates and cultured until confluent. Cells were washed twice with 3 ml of ice-cold PBS/CM (138 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 8.0). Biotinylation was carried out on ice by incubation with 1 ml of ice-cold PBS/CM containing a membrane-impermeable biotinylation reagent Sulfo-NHS-SS-biotin (0.5 mg/ml) (Pierce, Rockford, IL). After two successive 20 min incubations at 4^oC with freshly prepared NHS-SS-biotin and gentle shaking, cells were briefly rinsed with 3 ml of PBS/CM containing 100 mM glycine. Cells were further incubated at 4^oC with the same solution for 20 min to ensure complete quenching of the unreacted NHS-SS-biotin. Cells were then solubilized on ice by incubating in 1 ml of lysis buffer containing 20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethyl-sulfonyl fluoride and Protease Inhibitors Cocktail (Roche) for 1 h with occasional vortexing. Protein concentrations were measured from the supernatant lysate and fifty microliters of UltraLink Immobilized NeutrAvidin protein (Pierce) was then added to the supernatant for the isolation of membrane proteins.

Membrane proteins were subjected to Western blot using a mouse monoclonal anti-yellow fluorescent protein antibody (JL-8) (BD Biosciences) at 1:1000 dilution, followed by horseradish peroxidase-conjugated goat anti-mouse IgG (1:20,000 dilution). The chemiluminescent signals in the Western blots were detected by using SuperSignal West Pico Chemiluminescent Substrate (Pierce) followed by exposure of the blots to x-ray films. Band intensity was quantified by densitometry using the ImageQuant software (Molecular Dynamics). As reported previously (Zhou et al., 2007), double or multiple protein bands around the expected molecular size (~75 kDa) were observed for the YFP tagged PMAT proteins, which could be due to differential glycosylation of PMAT.

Data Analysis

For all uptake experiments, data were expressed as the mean ± S.D. from three independent experiments (n=3) with different cell passages. For each experiment, uptake was carried out in triplicates in three different wells on the same plate. Where applicable, *p* values were obtained through Student's t-test.

Whole exome sequencing and sequence analysis

Whole exome sequencing was carried out by Andrea Patrignani and Dr. Remy Bruggmann in collaboration with the Functional Genomics Center Zürich (FGCZ). The color space sequence reads (paired end 50x35) were mapped to the human genome assembly hg19 (ftp://hgdownload.cse.ucsc.edu/goldenPath/hg19/bigZips/chromFa.tar.gz) using the software Bioscope 1.2.1 from Applied Biosystems (Life Technologies, Carlsbad, CA, USA). After mapping, the single nucleotide polymorphisms (SNPs) were called by using the DiBayes algorithm (Life Technologies, Carlsbad, CA, USA) with high and medium stringency settings. The obtained SNPs were classified into intronic, intergenic, coding (synonymous and non-synonymous amino acid substitutions) and splice-site variants with a custom software pipeline. Furthermore, all SNPs were compared to dbSNP build 132, OMIM and clinically relevant mutation.

Search strategy and selection criteria

To analyse the whole exome sequencing data from the Functional Genomic Centre Zürich (FGCZ), we focused on candidate genes published to be involved in serotonin as well as candidate genes involved in autism spectrum disorders (ASDs). In order to find these candidate genes we first made a litterateur searched in Pubmed with the term: "serotonin" in combination with "synthesis", "metabolism", "transporter", "receptor", "transcription factor", "regulation". This was followed by a literature searched with the term: "autism" in combination with "candidate genes", "genes" and "genetics". These Pubmed search strategies led to the generation of two lists: one containing the candidate genes of interest published to be involved in serotonin (see table S2) and one with the candidate genes of interest involved in autism, ASDs and ASD-related syndromes (see table S3).

Screening of mutations in human GFRP gene

The following intronic primers were used to screen exon 1-3 in the human GFRP gene: For exon 1: GFRP5, 5'-TCCTCCCCGCACTCCACCTT-3' (forward) and GFRP6: 5'-CTCC TGTCCTACCTGTCGGC-3' (reverse), for exon 2: GFRP7, 5'-TCAAACTCCTCCCACCTC AA-3 (forward) and GFRP8: 5'-TAGAAGCAGGGAGCAGGGAG-3' (reverse), for exon 3: GFRP9, 5'-CTGGGACGCTGGGGAACCTG-3 (forward) and GFRP10: 5'-GTCAAGCAGT GGGAGAGGGC-3' (reverse). PCR reactions were performed in a final volume of 25 µl containing 1 µl of genomic DNA template (100 ng), 1 µl of each primer (5 µM), 0.5 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.5 µl Hot FirePol Hotstart DNA polymerase (Solis Biodyne), 1.5 µl 25mM $MgCl_2$, 2.5 µl 10x reaction buffer (Mg^{2+} free from Solis Biodyne) and 5 µl 5x Q-solution (Qiagen). The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95^{o}C for 15 minutes, followed by 35 cycles of denaturation 95°C for 60 seconds, annealing 54°C for 45 seconds and extension at 72°C for 30 seconds, a final extension was performed at 72°C for 10 minutes and termination at 4^{o}C. The same forward and reverse primers as mentioned above were used to sequence the amplified PCR products with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing PCR was performed in a 10 µl reaction mixture consisting of 0.5 µl PCR product, 0.8 µl primer (5 µM), 1.5 µl BigDye and 1.25 µl 5x sequencing buffer (Both BigDye and 5x sequencing buffer were from the BigDye Terminator v1.1 Cycle Sequencing Kit) using standard thermal cycling: (activation at 96^{o}C for 1 min, followed by 25 cycles of 96^{o}C for 10 seconds, 54^{o}C for 5 seconds, 60^{o}C for 1.5 minutes and termination at 4^{o}C). After end run 15 µl H_2O was added to the sequencing reactions before purified by gel filtration (using MultiScreen HV 96-well filter plates (Millipore) with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing results were compared with the reference sequence (Transcript ID: ENST00000260447 or accession number NM_005258.2) of the *GCHFR* gene.

Screening of 301 control subjects for CNTNAP2-N407S mutation

Intronic primers were designed to flank exon 8 in the human *CNTNAP2* gene: Forward: CNTNAP2_F: 5'-TTCATTTTATTCTGTGTTTTCCTCA-3' and reverse CNTNAP2_R: 5'-TGGAAATTGCAATAAGTTTCAGG-3'. PCR reactions were performed in a final volume of 25 µl containing 2 µl of genomic DNA template (100 ng), 0.5 µl of each primer (10 µM), 0.25 µl 10 mM deoxyribonucleoside triphosphates (dNTPs), 0.2 µl Hot FirePol Hotstart DNA

polymerase (Solis Biodyne), 1.5 µl 25mM $MgCl_2$ and 2.5 µl 10x Puffer B2 (Mg^{2+} free from Solis Biodyne). The PCR reactions were performed in a GeneAmp PCR System 9700 from Applied Biosystems using standard thermal cycling: hot start activation at 95°C for 15 minutes, followed by 35 cycles of denaturation 95°C for 60 seconds, annealing 54°C for 45 seconds and extension at 72°C for 30 seconds, a final extension was performed at 72°C for 10 minutes and termination at 4°C. The same forward and reverse primers as mentioned above were used to sequence the amplified PCR products with BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing PCR was performed in a 10.5 µl reaction mixture consisting of 0.8 µl PCR product, 0.8 µl primer (5 µM), 1.5 µl BigDye and 1.5 µl 5x sequencing buffer (BigDye and 5xsequencing buffer were both from BigDye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems)) using standard thermal cycling: (activation at 96°C for 1 min, followed by 25 cycles of 96°C for 10 seconds, 54°C for 5 seconds, 60°C for 1.5 minutes and termination at 4°C). After end run 15 µl H_2O was added to the sequencing reactions before purified by gel filtration (using MultiScreen HV 96-well filter plates (Millipore) with Sephadex G-50 (GE Healthcare)) and analyzed on a 3130*xl* Genetic Analyzer (Applied Biosystems). Sequencing results were compared with the reference sequence (Transcript ID: ENST00000297195 or accession number NM_001040661) of the CNTNAP2 gene.

5.5 Case report

Case report (MT)

A now 11 year old boy was born after an uncomplicated pregnancy, delivery and neonatal period. Both parents are known to suffer from anxiety disorder since childhood but do not have further health problems. His father works as a bank director and his mother used to work as a nurse in an infirmary. For his anxiety disorder paroxetine, a SSRI drug was prescribed to the father. The mother takes sertraline (an antidepressant of the selective serotonin reuptake inhibitor (SSRI) class). A 5 year old younger sister is healthy. The psychomotor and speech development were normal in the patient. However, the parents noticed that he did not show a normal imitative play and from early infancy, he suffered from sleeping problems due to anxiety. When he started kindergarten at the age of 2 ½ years he was described as a nervous child who did not seeking contact with other children, was found to be “egocentric”, did not play with fantasy, did not like changes and responded with anxiety, but showed restrictive interests in busses and trains.

He had few stereotypic movements like putting his hands in front of his mouth with an anxious expression. He had an extreme phobia for insects. From the age of 5 years he was seen by a child psychiatrist who suspected Asperger's syndrome with attention deficit disorder and prescribed methylphenidate in combination with risperidone at night. Formal testing and observation at home and in school at the age of nearly 9 years was performed by the centre for autism, using DSM-IV criteria, neuropsychological tests, evaluation of socio-emotional comprehension, Childhood Autism Rating Scale (CARS), and the Childhood Apperception Test (CAP). His physical and neurologic examinations were completely normal. In conclusion, he was found to fulfil all criteria for Asperger's syndrome with a CARS score of 39.5, indicating moderate to severe autism.

After informed consent, laboratory investigations at the age of 9 ½ years revealed normal routine tests including amino-acid levels. The only abnormality observed was a moderately diminished serum gamma-tocopherol. In addition spinal fluid analysis showed a low serotonine metabolite 5-HIAA at 65 nmol/L (normal range: 88-178 (Blau et al., 2003)), a normal HVA concentration at 389 nmol/L (reference: 144-801) with an elevated HVA/5-HIAA ratio at 6 (normal value 1.5-3.5). Spinal fluid concentrations for L-dopa, 5-hydroxytryptophane, pterins and L-N^5-methyltetrahydrofolate were normal. The whole blood serotonin level was slightly elevated at 287 ng/ml (normal range 50-200 (Burtis and Ashwood, 2001)) and platelet serotonin content was elevated at 1041 ng/10^9 platelets (normal range: 125-500 (Burtis and Ashwood, 2001).

Case report (PL)

A now 8 year old boy was born by caesarean delivery after a pregnancy of 37 weeks, complicated by maternal hypertension and gestational diabetes. His birth weight was 3.7 kg. His parents were healthy and non-consanguineous. He had an 11 month older sister in good health. After a normal neonatal period he was described as an inactive baby never crying that could be put anywhere. Developmental motor milestones were attained normally but as he started toe-walking from the age of 2 years, he would frequently fall. From the age of 2 years, he started to have severe eating problems and would only eat biscuits, chicken and French fries. During his first two years, he did not respond to his name, never pointed or attracted joint attention; he never waved good-bye and showed restrictive interests with absent imitative play. From the age of 3 years, he developed stereotypic patterns like running in circles, turning light switches on and off, balancing movements and head banging against the floor.

Because of serous middle ear effusions and suspected hearing loss at the age of two years, bilateral tympanic drains were placed but postoperatively there was no improvement of his response to external stimuli and speech did not develop.

He could utter single word fragments using syllables from the age of 3 years and developmental dysphasia was suspected. From the age of 4 ½ years he was transferred from kindergarten to a special school for mentally handicapped children.

The Autism Diagnostic Interview for parents (ADI-R) revealed that he fulfilled all criteria of infantile autism between the age of 4 and 5 years. Physical examination did not show any dysmorphic features and neurological status was completely normal. Twenty-four EEG monitoring and brain MRI were found normal.

Laboratory investigations at the age of 6 years revealed normal CSF cell counts, protein and glucose levels. Spinal fluid analysis showed diminished concentrations for the serotonin end-metabolite 5-HIAA but normal levels for the dopamine end-metabolite HVA, intermediate metabolites L-dopa, 5-hydroxytryptophane, pterines and L-N^5-methyl- tetrahydrofolate. Serum amino-acids were normal including L-tryptophane. The whole blood serotonin level was slightly elevated at 221 ng/ml (normal range 50-200 (Burtis and Ashwood, 2001)) and platelet serotonin content was elevated at 675 ng/10^9 platelets (normal range: 125-500 (Burtis and Ashwood, 2001)). Biochemical blood examination revealed slight deficiencies for serum and red blood cell folate content, as well as vitamin C, D, gamma-tocopherol, beta-carotene, zinc and selenium. He received vitamin and trace element supplements to correct these alimentary deficiencies.

From the age of 6 years he visited a special institute for autistic children and received re-education by the TEACCH method (**T**reatment and **E**ducation of **A**utistic and **C**ommunication related handicapped **CH**ildren) and using pictogrammes. Follow-up assessment at the age of 7 4/12 years using the ADI-R, Autism Diagnostic Observation Scale (ADOS) as well as observation at home and school, revealed moderate improvement with respect to non-verbal communication and behaviour, so that he now fulfilled the criteria for atypical autism (PDD-NOS).

Table S2: Candidate genes involved in serotonin synthesis, metabolism, transport, recycling, receptor, transcription factor and regulation

Protein name	Gene name	RefSeq IDs	OMIM Number	Function of gene	Reference
Tryptophan hydroxylase 1	*TPH1*	NM_004179	191060	Serotonin synthesis	[1]
Tryptophan hydroxylase 2	*TPH2*	NM_173353	607478	Serotonin synthesis	[1]
Aromatic L-amino acid decarboxylase (AADC)	*DDC*	NM_000790	107930	Serotonin synthesis	[1]
Aldehyde dehydrogenase 2 family (mitochondrial)	*ALDH2*	NM_000690	100650	Serotonin metabolism	[2]
Serotonin re-uptake transporter (SERT, 5-HTT)	*SLC6A4*	NM_001045	182138	Serotonin transport	[3]
Plasma membrane monoamine transporter (PMAT)	*SLC29A4*	NM_153247	609149	Serotonin transport	[4]
Organic cation transport 1 (OCT1)	*SLC22A1*	NM_003057	602607	Serotonin transport	[5]
Organic cation transport 3 (OCT3)	*SLC22A3*	NM_021977	604842	Serotonin transport	[5]
Vesicular monoamine transporter 2 (VMAT2)	*SLC18A2*	NM_003054	193001	Serotonin recycling	[3]
Monoamine oxidase A (MAO-A)	*MAOA*	NM_000240	309850	Serotonin receptor	[3]
Monoamine oxidase B (MAO-B)	*MAOB*	NM_000898	309860	Serotonin receptor	[3]
Serotonin receptor 1A	*HTR1A*	NM_000524	109760	Serotonin receptor	[3]
Serotonin receptor 1B	*HTR1B*	NM_000863	182131	Serotonin receptor	[3]
Serotonin receptor 1D	*HTR1D*	NM_000864	182133	Serotonin receptor	[3]
Serotonin receptor 1E	*HTR1E*	NM_000865	182132	Serotonin receptor	[3]
Serotonin receptor 1F	*HTR1F*	NM_000866	182134	Serotonin receptor	[3]
Serotonin receptor 2A	*HTR2A*	NM_000621	182135	Serotonin receptor	[3]
Serotonin receptor 2B	*HTR2B*	NM_000867	601122	Serotonin receptor	[3]
Serotonin receptor 2C	*HTR2C*	NM_000868	312861	Serotonin receptor	[3]
Serotonin receptor 3A	*HTR3A*	NM_000869	182139	Serotonin receptor	[3]
Serotonin receptor 3B	*HTR3B*	NM_006028	604654	Serotonin receptor	[3]
Serotonin receptor 3C	*HTR3C*	NM_130770	610121	Serotonin receptor	[3]
Serotonin receptor 3D	*HTR3D*	NM_182537	610122	Serotonin receptor	[3]
Serotonin receptor 3E	*HTR3E*	NM_182589	610123	Serotonin receptor	[3]
Serotonin receptor 4	*HTR4*	NM_000870	602164	Serotonin receptor	[3]
Serotonin receptor 5A	*HTR5A*	NM_024012	601305	Serotonin receptor	[3]
Serotonin receptor 5B	*HTR5B*	NG_008325	-	Serotonin receptor	[3]
Serotonin receptor 6	*HTR6*	NM_000871	601109	Serotonin receptor	[3]
Serotonin receptor 7	*HTR7*	NM_000872	182137	Serotonin receptor	[3]

Table S2: Candidate genes involved in serotonin synthesis, metabolism, transport, recycling, receptor, transcription factor and regulation

Protein name	Gene name	RefSeq IDs	OMIM Number	Function of gene	Reference
cAMP responsive element binding protein 1	*CREB1*	NM_134442	123810	Transcription factor	[3]
S100 calcium binding protein, beta	*S100β*	NM_006272	176990	Transcription factor	[3]
PET-1 (Fev-pending)	*FEV*	NM_017521	607150	Transcription factor	[3]
NK2 homeobox 2	*NKX2-2*	NM_002509	604612	Transcription factor	[6]
LIM homeobox transcription factor	*LMX1B*	NM_001174146	602575	Transcription factor	[6]
GATA binding protein 2	*GATA2*	NM_032638	137295	Transcription factor	[6]
GATA binding protein 3	*GATA3*	NM_001002295	131320	Transcription factor	[6]
Achaete-scute complex homolog 1 (Drosophila)	*ASCL1*	NM_004316	100790	Transcription factor	[7]
Paired-like homeobox 2b	*PHOX2B*	NM_003924	603851	Transcription factor	[6]
Homeobox B1	*HOXB1*	NM_002144	142968	Transcription factor	[6]
Homeobox B2	*HOXB2*	NM_002145	142967	Transcription factor	[6]
NK6 homeobox 1	*NKX6-1*	NM_006168	602563	Transcription factor	[6]
NK6 homeobox 2	*NKX6-2*	NM_177400	605955	Transcription factor	[6]
Orthodenticle homeobox 2	*OTX2*	NM_021728	600037	Transcription factor	[6]
GLI family zinc finger 1	*Gil1*	NM_005269	165220	Transcription factor	[6]
GLI family zinc finger 2	*GIL2*	NM_005270	165230	Transcription factor	[6]
Fibroblast growth factor 4	*FGF4*	NM_002007	164980	Transcription factor	[6]
Fibroblast growth factor 8	*FGF8*	NM_006119, NM_033165	600483	Transcription factor	[6]
Sonic hedgehog	*SHH*	NM_000193	600725	Transcription factor	[6]
Smoothened homolog (Drosophila)	*SMO*	NM_005631	601500	Transcription factor	[6]
Gastrulation brain homeobox 2	*GBX2*	NM_001485	601135	Transcription factor	[8]
GTP cyclohydrolase I feedback protein (GFRP)	*GCHFR*	NM_005258	602437	Regulator	[9]

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Table S3: Candidate genes published to be associated with autism, autism spectrum disorders (ASDs) and ASD-related syndromes

Protein name	Gene name	RefSeq IDs	OMIM Number	Association	Reference
Ataxin 2-binding protein	*A2BP1*	NM_018723	605104	ASDs	[1]
Adenosine deaminase	*ADA*	NM_000022	608958	Autism	[2]
Adenylosuccinate lyase	*ADSL*	NM_000026	608222	Autism	[2]
Abelson helper intergration site 1	*AHI1*	NM_017651	608894	ASDs	[1]
Aldolase A, fructose-biphosphate	*ALDOA*	NM_000034	103850	Autism	[3]
Amyloid beta precursos protein-binding, family A, member 2	*APBA2*	NM_005503	602712	Autism	[4]
Apolipoprotein E	*APOE*	NM_000041	107741	Autism	[2]
Androgen receptor	*AR*	NM_000044	313700	Autism	[2]
Aristaless related homeobox	*ARX*	NM_139058	300382	Autism	[2]
Activating transcription factor 2	*ATF2*	NM_001880	123811	Autism	[2]
ATPase, class V, type 10A	*ATP10C*	NM_024490	605855	Autism	[2]
Arginine vasopressin receptor 1A	*AVPR1a*	NM_000706	600821	Autism/ASDs	[2]
Brain-derived neurotrophic factor	*BDNF*	NM_170735	113505	Autism	[2]
Calcium channel voltage-dependent L type alpha 1C subunit	*CACNA1C*	NM_000719	114205	ASDs/ASD-related syndromes	[1]
Ca2+-dependent activator protein for secretion 2	*CADPS2*	NM_017954	609978	ASDs	[1]
Centaurin gamma 2	*CENTG2*	NM_001037131	608651	ASD-related syndromes	[1]
Cystic fribrosis transmembrane conductance regulator	*CFTR*	NM_000492	602421	Autism	[4]
Chimerin (chimaerin) 1	*CHN1*	NM_001822	118423	Autism	[2]
Contactin associated protein-like 2	*CNTNAP2*	NM_014141	604569	ASDsASD-related syndromes	[1]
Coatomer protein complex, subunit gamma 2	*COPG2*	NM_012133	604355	Autism	[2]
Coractin binding protein 2	*CORTBP2*	NM_033427	609772	Autism	[5]
Carboxypeptidase A1 (pancreatic)	*CPA1*	NM_001868	114850	Autism	[2]
Carboxypeptidase A5	*CPA5*	NM_001127441	609561	Autism	[2]
Dopamine beta-hydroxylase	*DBH*	NM_000787	609312	Autism	[2]
Doublecortin	*DCX*	NM_178153	300121	Autism	[2]
Aromatic L-amino acid decarboxylase	***DDC***	**NM_000790**	**107930**	**Autism/Serotonin**	[2]
7-dehydrocholesterol reductase	*DHCR7*	NM_001360	602858	ASDs/ASD-related syndromes	[1]
Disrupted in schizophrenia 1	*DISC1*	NM_018662	605210	ASDs	[1]

Table S3: Candidate genes published to be associated with autism, autism spectrum disorders (ASDs) and ASD-related syndromes

Protein name	Gene name	RefSeq IDs	OMIM Number	Association	Reference
Distal-less homeobox 1	*DLX1*	XM_087198	600029	Autism	[2]
Distal-less homeobox 2	*DLX2*	NM_004405	126255	Autism	[2]
Distal-less homeobox 6	*DLX6*	NM_005222	600030	Autism	[2]
Double C2-like Domain-containing protein, Alpha	*DOC2A*	NM_003586	604567	Autism	[2]
Dopamine receptor D1	*DRD1*	NM_000794	126449	Autism	[2]
Dopamine receptor D2	*DRD2*	NM_000795	126450	Autism	[2]
Dopamine receptor D5	*DRD5*	NM_000798	126453	Autism	[2]
Engrailed homeobox 2	*EN2*	NM_001427	131310	Autism/ASDs	[2]
Fragil X retardation 1	*FMR1*	NM_002024	309550	Autism/ASDs/ASD-related syndromes	[2]
Forkhead box P2	*FOXP2*	NM_014491	605317	Autism	[2]
GABA A receptor, alpha 5	*GABRA5*	NM_000810	137142	Autism	[2]
GABA A receptor, beta 3	*GABRB3*	NM_000814	137192	Autism/ASDs	[2]
GABA A receptor, gamma 3	*GABRG3*	NM_033223	600233	Autism	[2]
Glutamate decarboxylase 1	*GAD1*	NM_000817	605363	Autism	[2]
Glycine receptor, alpha 2	*GLRA2*	NM_001118885	305990	Autism	[2]
Glutamate receptor ionotropic kainate 2	*GRIK2*	NM_001166247	138244	Autism/ASDs	[2]
Glutamate receptor, metabotropic 3	*GRM3*	NM_000840	601115	Autism	[5]
Glutamate receptor, metabotropic 8	*GRM8*	NM_000845	601116	Autism	[2]
Gastrin-releasing peptide receptor	*GRPR*	NM_005314	305670	Autism	[2]
Hect domain and RLD 2	*HERC2*	NM_004667	605837	Autism	[5]
HIRA interacting protein 3	*HIRIP3*	NM_003609	603365	Autism	[3]
Homeobox A1	*HOXA1*	NM_005522	142955	Autism	[2]
Homeobox B1	*HOXB1*	NM_002144	142968	Autism	[2]
Homeobox D1	*HOXD1*	NM_024501	142987	Autism	[5]
v-Ha-ras Harvey rat sarcoma viral oncogene homolog	*HRAS1*	NM_176795	190020	Autism	[2]
Serotonin receptor 2A	***HTR2A***	**NM_000621**	**182135**	**Autism/Serotonin**	[2]
Serotonin receptor 7	***HTR7***	**NM_000872**	**182137**	**Autism/Serotonin**	[2]
Inositol polyphosphate-1-phosphatase	*INPP1*	NM_001128928	147263	Autism	[2]
Integrin, beta 3	*IGB3*	NM_000212	173470	ASDs	[1]
Laminin, beta 1	*LAMBI*	NM_002291	150240	Autism	[2]
Leucine rich repeat neuronal 3	*LRRN3*	NM_018334	-	Autism	[2]
Monoamine oxidase A	***MAOA***	**NM_000240**	**309850**	**Autism/Serotonin**	[2]
Monoamine oxidase B	***MAOB***	**NM_000898**	**309860**	**Autism/Serotonin**	[2]

Table S3: Candidate genes published to be associated with autism, autism spectrum disorders (ASDs) and ASD-related syndromes

Protein name	Gene name	RefSeq IDs	OMIM Number	Association	Reference
MYC-associated zinc finger protein	*MAZ*	NM_002383	600999	Autism	[3]
Methyl CpG binding protein 2	*MECP2*	NM_004992	300005	Autism/ASDs/ASD-related syndromes	[2]
Mesoderm specific transcript homolog	*MEST*	NM_002402	601029	Autism	[2]
Met proto-oncogene	*MET*	NM_000245	164860	ASDs	[1]
Neurobeachin	*NBEA*	NM_015678	604889	Autism	[5]
Necdin	*NDN*	NM_002487	602117	Autism	[5]
Neurogenic differentiation 1	*NEUROD1*	NM_002500	601724	Autism	[2]
Neurofibromin 1	*NF1*	NM_000267	613113	Autism	[2]
Neurofibromin 2	*NF2*	NM_000268	607379	Autism	[2]
Neuroligin 3	*NLGN3*	NM_018977	300336	Autism/ASDs	[2]
Neuroligin 4, X-linked	*NLGN4X*	NM_020742	300427	ASDs	[2]
Notch 4	*NOTCH4*	NM_004557	164951	Autism	[2]
Neuronal cell adhesion molecule	*NR-CAM*	NM_001037132	601581	Autism	[2]
Neurexin1	*NRXN1*	NM_001135659	600565	ASDs	[1]
Oxytocin receptor	*OXTR*	NM_000916	167055	ASDs	[1]
Piccolo (presynaptic cytomatrix protein)	*PCLO*	NM_014510	604918	Autism	[2]
Proprotein convertase subtilisin/kexin type 2	*PCSK2*	NM_002594	162151	Autism	[2]
Prodynorphin	*PDYN*	NM_001190892	131340	Autism	[2]
Proenkephalin	*PENK*	NM_001135690	131330	Autism	[2]
Protein phosphatase 4, catalytic subunit	*PPP4C*	NM_002720	602035	Autism	[3]
Phosphatase and tensin homologue	*PTEN*	NM_000314	601728	ASDs	[1]
RAB3A, member RAS oncogene family	*RAB3A*	NM_002866	179490	Autism	[2]
Reelin	*RELN*	NM_005045	600514	Autism/ASDs	[2]
Sodium channel, voltage-gated, type I, alpha subunit	*SCN1A*	NM_006920	182389	Autism	[2]
Sodium channel, voltage-gated, type II, alpha subunit	*SCN2A*	NM_021007	182390	Autism	[2]
Sodium channel, voltage-gated, type III, alpha subunit	*SCN3A*	NM_006922	182391	Autism	[2]
Secretin	*SCT*	NM_021920	82099	Autism	[2]
Mitochondrial aspartate/glutamate carrier (AGC1)	*SLC5A12*	NM_003705	603667	Autism/ASDs	[2]
Mitochondrial aspartate/glutamate carrier citrin	*SLC13A13*	NM_014251	603859	Autism	[6]

Table S3: Candidate genes published to be associated with autism, autism spectrum disorders (ASDs) and ASD-related syndromes

Protein name	Gene name	RefSeq IDs	OMIM Number	Association	Reference
Serotonin re-uptake transporter	***SLC6A4***	**NM_001045**	**182138**	**Autism/ASDs/Serotonin**	[2]
Seizure related 6 homolog (mouse)-like 2	*SEZ6L2*	NM_012410	-	Autism	[3]
Small nuclear ribonucleoprotein polypeptide N	*SNRPN*	NM_003097	182279	Autism	[5]
SH3 and multiple ankyrin repeat domains 2	*SHANK2*	NM_012309	603290	ASDs/ASD-related syndromes	[7]
SH3 and multiple ankyrin repeat domains 3	*SHANK3*	NM_001080420	606230	ASDs/ASD-related syndromes	[2]
Somatostatin receptor 5	*SSTR5*	NM_001053	182455	Autism	[2]
Suppression of tumorigenicity 7	*ST7*	NM_021908	600833	Autism	[5]
TAO kinase 2	*TAOK2*	NM_016151	613199	Autism	[2]
T-box, brain, 1	*TBR1*	NM_006593	604616	Autism	[2]
Tryptophan 2,3-dioxygenase	*TDO2*	NM_005651	191070	Autism	[2]
Tyrosine Hydroxylase	*TH*	NM_000360	191290	Autism	[2]
Tuberous sclerosis 1	*TSC1*	NM_000368	605284	Autism/ASDs/ASD-related syndromes	[2]
Tuberous sclerosis 2	*TSC2*	NM_021055	191092	Autism/ASDs/ASD-related syndromes	[2]
Ubiquitin-conjugating enzyme E2H	*UBE2H*	NM_003344	601082	Autism	[2]
Ubiquitin protein ligase E3A	*UBE3A*	NM_000462	601623	Autism/ASDs/ASD-related syndromes	[2]
Vasoactive intestinal peptide receptor 2	*VIPR2*	NM_003382	601970	Autism	[5]
Wingless-type MMTV integration site family member 2	*WNT2*	NM_003391	147870	Autism	[2]

* In bold are the candidate genes which have been published to be both serotonin and autism/ASDs/ASD-related syndromes.

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PKU-BH_4 meeting, March 2008, St. Mortiz, Switzerland.

Dea Adamsen, Rossana Scavelli, Nenad Blau and Beat Thöny

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"Generation of a new mouse model for BH_4 deficiency by knocking-in a single codon exchange (R15C) in the BH_4 biosynthetic gene 6-pyruvoyltetrahydropterin synthase (*Pts*)"

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ZNZ symposium. September 2010, Zürich.

Dea Adamsen, Rossana Scavelli, Birgit Ledermann, Nenad Blau and Beat Thöny

"A murine *Pts* model to study its role in BH_4 deficiency and monoamine neurotransmitter function and contribution to human "

Dea Adamsen, David Meili, Nenad Blau, Vincent Ramaekers and Beat Thöny

"Atypical autism associated with the heterozygous Gly56Ala allele of the *SLC6A4* serotonin transporter gene and low serotonin turnover in CNS"